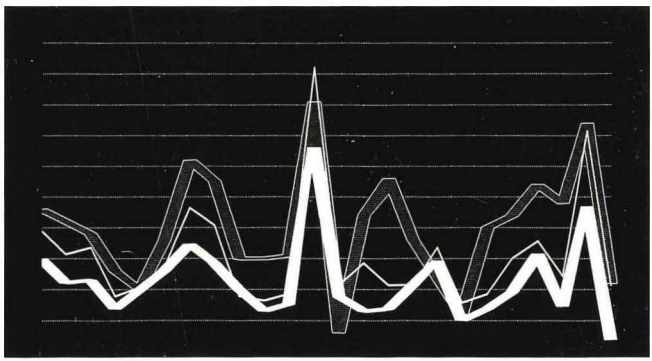
PATHOGENESIS OF TESTICULAR GERM CELL TUMORS. A CYTOGENETICAL AND PATHOLOGICAL STUDY.



SÉRGIO CASTEDO

PATHOGENESIS OF TESTICULAR GERM CELL TUMORS. A CYTOGENETICAL AND PATHOLOGICAL STUDY.

STELLINGEN

I. The classic model of tumor progression proposed by Nowell does not apply to testicular germ cell tumors.
(in this thesis)

II. It is tempting to call orchidoblastomas extragonadal tumors of the testis.
(in this thesis)

III. Fusion of a post-meiotic haploid with a diploid cell may play an important role in the oncogenesis of testicular germ cell tumors of the adult.
(in this thesis)

IV. The i(12p) may probably be considered the "Philadephia chromosome of germ cell tumors".

V. The present scientific enthusiasm concerning tumor suppressor genes has had its parallel some years ago with the "oncogene fashion".

VI. Mitotic recombination may account for the apparent polyclonal origin of some malignancies.
(Stamberg J. and Hirschfield L. Cancer Genet Cytogenet 27: 5-8,1987)

VII. The frustrating side of dysmorphology is that in only around 50% of all dysmorphic patients a specific diagnosis can be reached. The optimistic side is that in those cases where no specific diagnosis can be established the empiric recurrence risk is relatively low (3-5%).

VIII. Practically speaking, if timesaving devices really saved time, there would be more time available to us than ever before in history. But, strangely enough, we seem to have less time than even a few years ago. (...) You can't save time. You can only spend it. (...) By trying to save every bit of it, one ends up wasting the whole thing.
(In: The Tao of Pooh, Benjamin Hoff)

IX. There are three kinds of lies: lies, damned lies, and statistics.
(Disraeli)

X. The scientists were wrong... the most persistent principles of the Universe were accidental errors.
(In: Dune, Frank Herbert)

XI. Hofstadter's law: It always takes longer than you expect, even when you take into account Hofstadter's law.

Stellingen behorend bij het proefschrift van Sérgio M. M. J. Castedo
(Groningen, 15 Juni 1988)

RIJKSUNIVERSITEIT GRONINGEN

PATHOGENESIS OF TESTICULAR GERM CELL TUMORS. A CYTOGENETICAL AND PATHOLOGICAL STUDY.

PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit Groningen
op gezag van de Rector Magnificus Dr. S. K. Kuipers
in het openbaar te verdedigen op woensdag 15 juni 1988
des namiddags te 4.00 uur
door

SÉRGIO MANUEL MADEIRA JORGE CASTEDO

geboren te Oporto (Portugal)

1988
DRUKKERIJ VAN DENDEREN B.V.
GRONINGEN

Promotores: Prof. Dr. J. W. Oosterhuis
Prof. Dr. C. H. C. M. Buys

Referent: Dr. Bauke de Jong

Promotiecommissie: Prof. Dr. D. Bootsma
Prof. Dr. J. D. Elema
Prof. Dr. H. Schraffordt Koops

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To Raquel

A tree as big around as you can reach starts with a small seed.
A thousand-mile journey starts with one step.

(Lao-Tse)

What I like doing best is Nothing...

CONTENTS

FOREWORD

When I first came to Groningen (September/1985) to meet Bauke and discuss the possibilities of doing here the work for my PhD, I was feeling a bit disoriented, like Alice in Wonderland:

"Would you tell me, please, which way I ought to go from here?," Alice (i.e. I) asked.

"That depends a good deal on where you want to get to," said the Cat (i.e. Bauke).

"I don't much care where -," said Alice.

"Then it doesn't matter which way you go," said the Cat.

"So long as I get somewhere," Alice added as an explanation.

"Oh you're sure to do that," said the Cat, "if you walk long enough."

After a brief hesitation, I decided to come to The Netherlands. It wasn't an easy step, though. Behind, Raquel and I would leave the family, the friends, and... the sun, and the blue sky.

Less than two years have elapsed - happily, Hofstadter's law didn't apply so well in this case... -, and never we regretted our decision. The work was accomplished, that means, I got somewhere, as Alice would say.

This was made possible by the contribution of several people, whom I would like to mention:

First Prof. Dr. Amândio S. Tavares, my director in Portugal, who allowed and supported my coming here.

I am very grateful to Wolter for having introduced me to such a fascinating subject. From you I will recall the image of a bright scientist, aware of how fruitful a cooperation can be with someone with complementary knowledge, like Bauke. I also thank Charles for all his useful criticisms and suggestions concerning my work, and for always finding some time for me, in spite of his overbooked "agenda". I would also like to thank the members of the "promotie commissie" (Prof. D. Bootsma, Prof. J.D. Elema, and Prof. H. Schraffordt Koops) for their willingness to read this thesis in such a short time.

I also gratefully acknowledge the Jan Kornelis de Cock Stichting and the Hubrecht Laboratorium, Utrecht, for financial support.

Then, Menke. Dear Menke, I really appreciated your promptness in making the photos I needed, as well as your understanding about the mess I have lately made of your room. Also René was always available to try again and again to make the nice graphs that illustrate my thesis - thank you very, very much!

To Jorge and the so-called "Mimis" (Mary, Paula, Salomé, and Cecília) goes my gratitude for keeping in touch, for support, and for making us believe that "there is no such thing as far away".

Now my "paranymphen". Dear AnneMarie, in you we have had a true friend from the very beginning and we will never forget the nice time we had together, travelling, skating (i.e. you skating, I falling...), working, or just "kletsen". Dear Marjolijn, with you I learned a little Dutch (maar toch spreek ik altijd engels...), but mainly that we can be good friends even without being often together. Both of you (AnneMarie and Marjolijn) I thank for being practically the only people that knocked at our door without appointment, and for all the arrangements you made for my thesis (though I still don't know what's going on!...).

My very special gratitude goes to my parents, for all their support during these (almost) 2 years. How nice it was to receive regularly some delicious Portuguese delicatessen!

From my parents-in-law and Zé Paulo I recall their encouragement, and the (almost) permanent telephone line between Porto and Groningen, through which we could keep aware of the local news. Both my parents and my parents-in-law we thank for always having our house so nice when we'd arrive.

A very special word goes to Bauke, my "referent". In spite of my former difficulties in communicating with you, I soon got used to your economy of words and straightforwardness. In you I appreciated not only your solid knowledge on the field of tumor cytogenetics, but also your reliability, your friendly help, and your trusting me. I will really miss this Fries!...

At this time of the foreword, I realize it is common to thank the wife for understanding, patience, and support. I'd rather thank you, Raquel, for your impatience, your help in the work, and your sacrifice leaving Portugal. None of this work would have been possible in such a short time without you.

THANK YOU ALL!!!

CHAPTER I

GENERAL INTRODUCTION

PATHOGENESIS OF TESTICULAR GERM CELL TUMORS

A. PRIMARY TESTICULAR GERM CELL TUMORS

Testicular germ cell tumors of adults can be divided both clinically and morphologically in two distinct entities, seminoma and nonseminoma [1-3]. The latter may have one or more of the following histological subtypes: embryonal carcinoma, teratoma, yolk sac tumor and choriocarcinoma. In the British classification [4] tumors with a seminoma and a nonseminoma component are classified as combined tumors, in the WHO classification as nonseminomas [3]. In about 20% of germ cell tumors seminoma and nonseminoma coexist [4,5]. Seminomas are less aggressive than nonseminomas as a group (although the aggressiveness of nonseminomas depends on the histological subtype, in particular the presence of embryonal carcinoma, yolk sac tumor and/or choriocarcinoma). This is reflected in a lower stage at presentation for seminoma than for nonseminoma. Seminomas present in stage I in 50-60% of the cases at a mean age of about 40 years [6]. Nonseminomas present in stage I in about 25% of the cases at a mean age of about 30 years (see [7] for review). Patients with combined tumors are slightly younger than patients with seminomas, but older than those with nonseminomas [5]. Testicular tumors in the pediatric age group are probably best considered as a distinct subgroup because of their epidemiology [8], clinical behavior (80% in stage I), and the distribution of morphological subtypes [9-11]. The majority is pure yolk sac (orchidoblastoma) or pure teratoma [9-11]. In this age group seminomas and mixed tumors are extremely rare [9-11], and in situ carcinoma could not be demonstrated in the testicular parenchyma adjacent to the tumor [12]. Spermatocytic seminoma is an exceptional tumor as well. Epidemiologically, clinically and morphologically it differs from classic seminoma (see [13] for review). Moreover, carcinoma in situ does not seem to play a role in its pathogenesis [14].

There are in essence two main theories about the pathogenetic relationship between seminomas and nonseminomas (Figure 1). The one advocated by Mostofi and coworkers [15,16] among others, assumes that seminomas and nonseminomas derive independently from transformed (dysplastic) intratubular germ cells via carcinoma in situ. This model, an update of the former hypothesis of Pierce [17], is schematically shown in Figure 1-a).

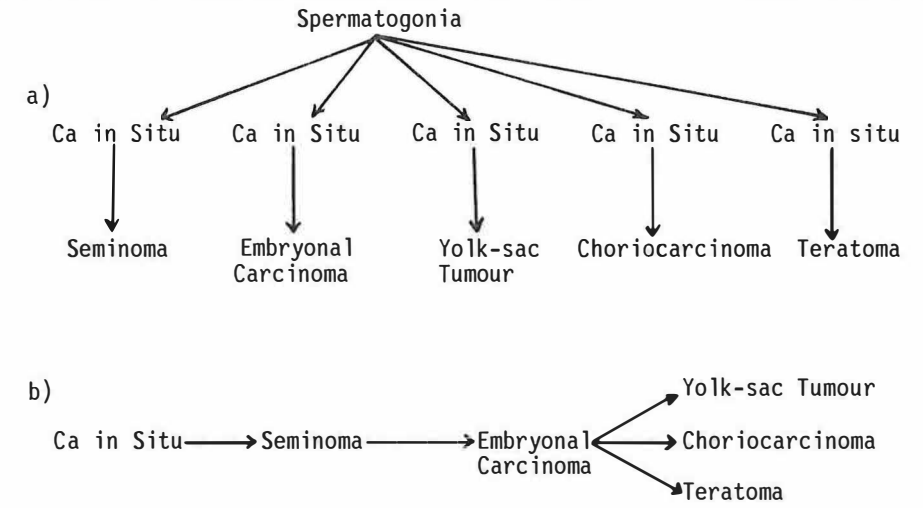


FIGURE 1. Models of origin of germ cell tumors of the testis.

A second theory, favored by Raghavan [18], Oliver [19] and by us, represents a further development of the earlier models of Ewing [20] and Friedman [21] and suggests that seminomas and nonseminomas have a single origin with seminoma as a stage after in situ carcinoma through which all germ cell tumors progress (Figure 1b).

As will be shown in this thesis we support the second view on ground of our studies about the cellular DNA content of in situ carcinoma and the different tumor subtypes, and their chromosomal pattern.

B. MATURE RESIDUAL TERATOMAS FOLLOWING POLYCHEMOTHERAPY

Untreated metastases of nonseminomatous germ cell tumors usually retain the morphologically appearance of the primary tumor (see [7] for review). As is the case in primary nonseminomas such metastases rarely consist exclusively of fully differentiated mature somatic tissue [22-25]. Recently, due to a variety of treatment schedules, containing polychemotherapeutic agents, irradiation or both, mature metastases are much more commonly encountered (see [7] for review). How this shift towards higher differentiation in the metastases of nonseminomatous testicular germ cell tumors is brought about is not known, although three possible mechanisms have been proposed:

a) Selective destruction of components other than mature teratoma [26-

29].

b) Direct induction of differentiation of malignant cells [27-30].

c) Spontaneous differentiation of the malignant cells made possible or facilitated by chemotherapy [29].

The mechanisms a) and c) are essentially similar and based on selection: there is selection of already existing mature teratoma in a) and of cells with inherent capacity of spontaneous somatic differentiation in c). Thus only two basically different mechanism remain in consideration: induction of differentiation or selection. These two mechanisms are not mutually exclusive.

It has been shown that mature residual tumor tissue following chemotherapy is significantly more often associated with primary tumors containing areas of mature teratoma than with primary tumors without mature teratoma [31,32]. Apparently the chemotherapy in order to produce mature residual tumor tissue needs tumor cells with an inherent capacity for somatic differentiation. The same was found in murine teratocarcinoma models. Only models with an inherent capacity for somatic differentiation give rise to mature residual tumor following treatment with cis-diammine-dichloroplatinum [33,34]. These findings support the contention that mature residual tumor tissue following chemotherapy is the result of selective survival of tumor cell clones with an inherent capacity for somatic differentiation and not the result of induction of differentiation in tumor cells (embryonal carcinoma cells) irrespective of their capacity for differentiation.

CYTOGENETIC AND PLOIDY STUDIES OF TESTICULAR GERM CELL TUMORS (REVIEW OF THE LITERATURE)

Early cytogenetic studies of testicular germ cell tumors described a generally hyperdiploid to hypertriploid chromosome complement with the modal chromosome number of seminomas higher (usually 60-69 chromosomes) than that of teratomas (50-59 chromosomes) [34-37], and combined tumors with intermediate modes [34]. Atkin [35] suggested the possibility that testicular tumors might arise from triploid rather than diploid or haploid cells. Wang et al. [38] reported cytogenetic evidence for premeiotic transformation of human testicular cancers. They observed simultaneous existence of X and Y chromosomes in the cells of 14 out of

15 tumor cell lines. They concluded their results to be most compatible with a diploid origin for these tumors. Recent studies [39-46] confirmed the early observations about chromosome numbers in the different testicular tumors. Atkin and Baker [46] found in ten seminomas chromosome numbers ranging from 55-105 (mean: 82). DeLozier-Blanchet et al. [42] found an average modal chromosome number for seminomas of 69, for an embryonal carcinoma it was 54, and for tumors of mixed histology it was 59. She found a significant higher number of chromosomes in seminomas as compared to nonseminomas. These findings were confirmed by DNA cytophotometric analysis. Gibas [41] found four embryonal carcinomas with an average chromosome number of 67, and two immature teratomas with an average number of 59. Martineau [17] found in combined tumors (histologically recognizable as seminoma and teratoma) that there was evidence that the two types of cells were chromosomally identical, or at least had related karyotypes. Her studies, however, are difficult to fully interpret, since they were carried out prior to the introduction of banding techniques. Berger [44] described a seminoma and an embryonal cell carcinoma occurring in different poles of the same testis with both tumors containing related karyotypes. She found in 6 tumors an overrepresentation of the chromosomes 2, 7, 8, 12, 14, 15, 16, 17 and 20. Atkin and Baker [39] found in their three seminomas an overrepresentation of the chromosomes 12, 19, 20, 21 and 22 and an underrepresentation of the chromosomes 11 and 13. Sandberg [45] described in a review of chromosomal abnormalities in testicular germ cell tumors in both malignant teratomas and seminomas a deficiency of group B and an excess of group C chromosomes. In seminomas an excess of group F and a deficiency of chromosomes 17 and 18 have been also reported (see [45] for review]. Comparing the ratios of various pairs of groups he described that pure tumors could be distinguished by the features of the various ratios of the different groups of chromosomes, and that combined tumors appear to occupy an intermediate position in this respect.

The most common structural abnormality found in testicular germ cell tumors is the isochromosome i(12p) [39-44,46-48]. This marker is characteristic for all histologic varieties of germ cell tumors of the testis [41,42]. Its occurrence in the various histological types of testicular germ cell tumors points to their pathogenetic interrelationship. A number of other, not consistent, structural chromosomal abnormalities are observed in testicular germ cell tumors. Delozier-Blanchet

[48] found in 23 tumors the chromosome regions most affected 12p, 17q, 1p and 1q, 9q, 22q, 6q and 7p. Abnormalities of chromosome 1 are often seen in testicular germ cell tumors [39,41,42,49-52], deletions of 1p being the most frequent [42]. Parrington et al. [50-52] have shown that cell lines established from testicular teratomas containing #1 rearrangements in addition to two apparently normal #1. The intact chromosomes #1 appeared to be identical. They also found homozygosity for the satellite region of chromosome 13. Atkin and Baker [39] found in each of three seminomas structural abnormalities of chromosome 1 and 12. The chromosome 1 changes involved duplication of 1q and loss of 1p. Apparently, events leading to loss of heterozygosity of 1p and 13q may be important in the oncogenesis of testicular germ cell tumors.

It seems very likely that the cell of origin of testicular germ cell tumors is polyploid [19,53,54] either triploid or tetraploid, resulting from either repeated nondisjunctions, polyploidization (see [45], for review) or fusion [55-57] of diploid with diploid or haploid cells. Polyploidization is a rather unique feature of testicular germ cell tumors. Most ovarian [58,59] and extra gonadal germ cell tumors (benign or malignant) are near diploid [60]. Gonocytes from which abnormal premalignant intratubular cells are derived, may be particularly prone to polyploidization, because they combine high mitotic activity and the phenomenon of bridge formation. In particular a defective mechanism of bridge formation has been implicated in the pathogenesis of germ cell tumors [61].

PROGRESSION OF TESTICULAR GERM CELL TUMORS

1. Progression model

In vivo hybridization or polyploidization could play a significant role in oncogenesis and neoplastic progression [35,45,55,56,62]. The existence of aneuploidy might provide a basis for continuing nondisjunction, and facilitate and heighten the genetic instability of neoplastic cells.

The tumor progression model of testicular cancers will probably consist of the following components:

1) Aneuploidy due to cell fusion or polyploidization.
2) Genetic instability due to aneuploidy [56,63].

3) Gain or retention of chromosomes or chromosomal material with a selective growth advantage. Development of structural chromosomal abnormalities and gene amplification [62-73].
4. Loss of chromosomes or chromosomal material where the loss will represent a selective growth advantage, for example by loss of tumor suppression [73-83] or loss of the ability of terminal differentiation [76,77].

It is generally accepted that progression of a malignant tumor is the result of clonal evolution of a tumor cell population, characterized by increasing aneuploidy and genetic instability of tumor cells, increasing proliferative potential, decreasing capacity of differentiation and higher malignant potential [62-66,68-71,77,78,84].

Contrary to the tumorprogression model originally proposed, and extended, by Nowell [64-66] in which the clonal evolution of a tumor cell population goes from diploid to hyperdiploid or highly aneuploid chromosome numbers, in testicular germ cell tumors of the testis tumor progression might go from high to lower numbers of chromosomes.

This would mean that besides gain of chromosomes or parts of chromosomes and development of structural chromosomal abnormalities, loss of chromosomes or chromosomal material must be a very early fundamental and important feature of tumorprogression of testicular germ cell tumors.

2. Tumorsuppression

Recent evidence shows that tumors may result from (functional) loss of both copies of certain gene loci. Loss of the normal allele by loss of a whole chromosome or a partial deletion will unmask a recessive mutant allele predisposing to cancer [81,83]. The best studied examples are retinoblastoma and Wilms tumor [80,82,85]. In these childhood cancers a predisposing mutation may also occur in the germ line. Therefore, hereditary forms occur besides sporadic ones. In the hereditary form multifocal tumors will be common since a tumor results from a single event - loss of the normal allele - which can occur in any cell. A number of retinoblastoma and Wilms tumor patients already have a constitutional visible chromosome deletion [86,87]. In the sporadic cases two events - loss of both normal alleles - have to coincide within the same cell in order to result into a tumor. Other tumors where a genetic component is clearly involved, like osteosarcoma and melanoma

show the same mechanism of inactivation and deletion of two gene copies [88,89]. Most interestingly, there are recent suggestions that the same holds true for tumors without any known genetic component, like bladder cancer [90] and lung cancer [91]. In the latter case, a visible chromosome deletion has been observed in tumor-derived cell lines and tumors [92].

Besides cytogenetic evidence in the form of chromosome deletions, and besides comparative DNA analyses of constitutional and tumor tissues showing loss of heterozygosity of certain DNA sequences in the tumors, cell fusion experiments have also contributed greatly to the concept of tumor suppression. When malignant cells are fused with normal cells of the same species the resulting hybrid cell is not malignant as long as some specific chromosomes are retained. When these particular chromosomes are eliminated malignancy will reappear [74-79]. Those chromosomes that, by elimination, give rise to reappearance of malignancy probably possess tumorsuppressing activity. Chromosomes important for tumorsuppression, as assessed by cell hybrid studies, are chromosome 11 [75,79,93,94] chromosome 14 [79,93] and 13 [75] and some combinations of chromosomes [75]. In the above referred models tumorsuppression appeared to be doses dependent [95-99], sometimes showing a kind of titration effect [97], and events leading to loss of heterozygosity were important [94,99].

Suppression, appearance and reappearance of malignancy associated with chromosome changes, including changes in gene balance has not only been found after hybridization between different types of cells but it also appears in malignant and non-malignant cells through chromosome segregation resulting in a change in gene dosage due to a change in the balance of specific chromosomes (see [100] for review). Sachs and coworkers (see [100] for review) showed in chromosome studies on normal fibroblasts, sarcomas, revertants from sarcomas which had regained a non-malignant phenotype, and re-revertants, that the difference between malignant and non-malignant cells is controlled by the balance between genes responsible for expression (oncogenes) and suppression (tumor suppressor genes) of malignancy and that this balance is disturbed by gain or loss of chromosomes or by processes leading to loss of heterozygosity.

The tumorsuppressor genes which are implicated in the genesis of human tumors as retinoblastoma and Wilms' tumor may represent a dif-

ferent class from those identified in somatic cell hybrids [97].

Apparently some chromosomes (for example chromosome 11) are more important for general tumorsuppression than other chromosomes, perhaps because they contain some "master" suppressor gene and/or are important for a broad spectrum of differentiation.

3. Relation between malignancy, tumorsuppression and differentiation.

Harris [76,77] developed a general model in which he proposed that the progressive multiplication of malignant cells is a secondary consequence of a genetically stable impairment of terminal differentiation. Harris [76,77], Sachs [100], Klinger [75] and Klein [71] propose that malignancy is a defect in normal-cell-function regulation and a defect in the capacity to differentiate to mature cells and that suppression of malignancy can be achieved by restoration of normal terminal differentiation, bringing cell multiplication to a stop, as a corollary, the most undifferentiated tumors have the most aggressive behavior. As for testicular germ cell tumors there is circumstantial evidence that metastatic lesions have a more limited repertoire of differentiation than the primary tumors [7].

So the chromosomes important for tumorsuppression must play some decisive role in normal differentiation.

OUTLINE OF THE THESIS

It is not unrealistic to think that the appearance of malignancy in dysplastic germ cell precursors of testicular germ cell tumors, and tumorprogression, after loss of chromosomes (=loss of tumorsuppression) is comparable with the loss of chromosomes from normal cells, malignant cells and somatic cell hybrids, as outlined above, by which malignancy will (re)appear.

Oncogenesis in testicular germ cell tumors may be a combination of loss of genes with tumorsuppressing and differentiation regulating properties, and gain of genes leading to tumorexpression or tumor progression.

Careful analysis of the numerical and structural abnormalities, and comparison of constitutional heterozygosity with tumor homozygosity will reveal which genomic events are important for tumorexpression and which

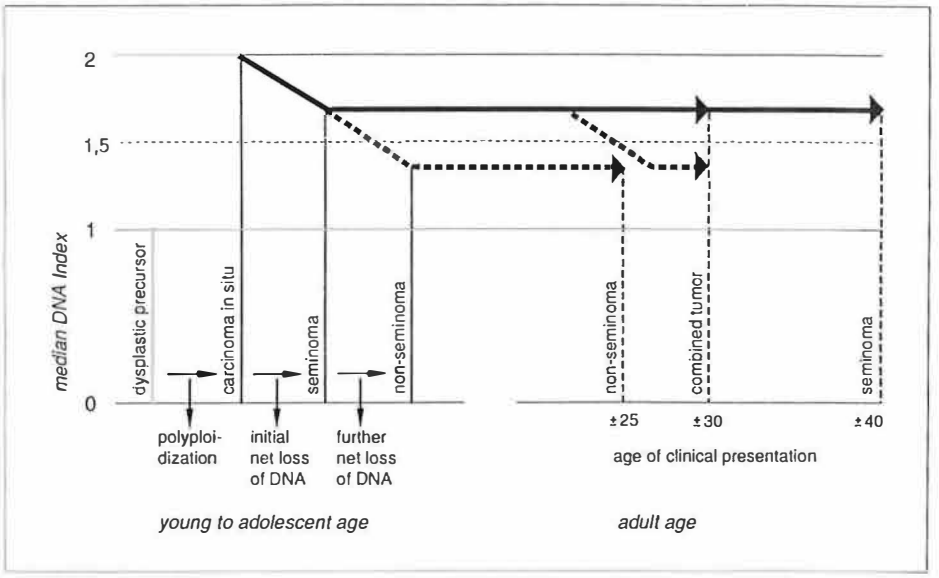
for tumorsuppression and normal differentiation.

If loss of chromosomes in testicular germ cell tumors is related to loss of genes crucial for normal cell differentiation, different chromosomes should be underrepresented in the different histological subtypes.

The malignant growth advantage obtained through the gain of chromosomes and through the evolvement of structural chromosomal abnormalities may also be tissue specific.

Accordingly, besides chromosomal abnormalities common to all germ cell tumors, in different histological subtypes different chromosomes should be over- and underrepresented, and different structural abnormalities should be present.

The progression model suggested here is in its most simple form shown in figure 2.



The purpose of the investigations reported in this thesis is to study in testicular germ cell tumors whether: 1. the karyotype evolution, accompanying the tumorprogression of testicular germ cell tumors, goes from high to lower numbers of chromosomes. 2. these tumors have a single origin with seminomas as a stage after in situ carcinoma through which all nonseminomas progress. 3. in different subtypes of these tumors different specific chromosomes, that may have tumor

suppression properties and play some decisive role in the normal differentiation, have been lost and are underrepresented. 4. in the different tumorsubtypes, besides common chromosomal abnormalities, different chromosomes will be overrepresented and specific structural chromosomal abnormalities will be present. 5. residual mature teratomas following polychemotherapy are the result of selection of less abnormal clones from the primary testicular nonseminoma, with the right balance of genes allowing somatic differentiation.

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CHAPTER II

PLOIDY OF SUBTYPES OF PRIMARY GERM CELL TUMORS OF THE TESTIS. PATHOGENETIC AND CLINICAL RELEVANCE.

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ABSTRACT

Testicular germ cell tumors (GCT) are a heterogeneous group of neoplasms of which the ploidy is not well established.

Using DNA flow cytometry, a significantly different median DI was found for orchidoblastomas (n=10), seminomas (n=20), and nonseminomas (n=36), of respectively: 1.91, 1.66 and 1.43.The seminoma and nonseminoma components of combined tumors (n=16) had a significantly different median DI of 1.61 and 1.40 respectively. Three of the 10 orchidoblastomas were diploid, compared to only one of the 72 testicular tumors of adults.

The data fit into a model of pathogenesis of testicular GCTs of adults in which all tumors, with the possible exception of spermatocytic seminoma, pass through a seminoma stage. Tumor progression seems to result from net loss of chromosomes from a (near)tetraploid carcinoma in situ cell. The pathogenesis of orchidoblastoma might be different from that of testicular GCTs of adults.

The consistent aneuploidy of testicular GCTs of adults might be helpful in making the differential diagnosis with primary non-germ cell tumors of the testis, and in differentiating between metastases of testicular GCTs and primary extragonadal malignant GCTs.

INTRODUCTION

The ploidy of germ cell tumors (GCTs) of the testis, in particular the proportion of diploid tumors is not well established. In flow-cytometric studies the percentage of diploid tumors ranges from about 5% to over 30% (14, 46, 52, 63). Atkin and Kay (4) using microdensitometry, studied 30 testicular GCTs and found few (near)diploid tumors. However, spermatocytic seminoma is often diploid (32, 54), and might have a pathogenesis, which is different from that of other GCTs of the testis (32).

Chromosome studies of testicular GCTs consistently show higher than normal chromosome numbers usually in the triploid range (1, 2, 3, 10, 18, 19, 28, 37, 60). Diploid testicular GCTs with clonal structural abnormalities of the chromosomes have not been reported.

We present the results of DNA flow cytometry of 10 orchidoblastomas, 20 seminomas, 36 nonseminomas, and 16 tumors with a seminoma and a nonseminoma component (combined tumors according to the British-classification), and discuss the relevance of the findings for the

understanding of the pathogenesis of these tumors.

Experimental design

Staging

Patients with testicular GCT were staged according to the system proposed by Peckham (42) using methods as described (16).

Histological classification

For every tumor the following histological components as defined by the WHO classification, were listed: seminoma, embryonal carcinoma, teratoma (immature and mature taken together), yolk sac tumor, and choriocarcinoma (30, 31). Minute foci of yolk sac-and trophoblastic differentiation which are frequently intermingled with other components were not listed separately. The tumors were grouped into four types (Table 1): orchidoblastoma (infantile embryonal carcinoma in the WHO classification), seminoma, nonseminoma and combined tumor.

Table 1. Patient material

Histology	Patients (n)	Clinical stage at presentation				Age in years average ± SD
		I	II	III	IV	
orchidoblastoma	10	9	0	1	0	1.8 ± 1.2
seminoma	20	11	7	1	1	39.0 ± 14.2
combined tumor	16	6	3	5	2	30.4 ± 7.4 #
non-seminoma	36	16	8	8	4	26.3 ± 7.1 #

#p<0.05, one tailed t-test.

The latter type consists of tumors containing a seminoma and a nonseminoma component, identical to the combined tumors defined by the British Testicular Tumor Panel (BTTP) classification (44). The seminomas were all of the classical type. The majority of the nonseminomas were mixed tumors. The composition of the nonseminomas is listed in Table 2, and of the combined tumors in Table 3.

Collection of material

Since orchidoblastomas are rare we had to rely on archival material. Nine cases were collected. One recent case was added in the course of the study. As combined tumors are also relatively rare, eight out of the 16 cases were archival cases.

Table 2. Histological composition of nonseminomas

Histology	n
EC	5
EC + T	19
EC + T + YO	1
EC + T + CH	1
EC + T + YO + CH	3
EC + YO	3
T	4

(EC = embryonal carcinoma; T = mature and immature teratoma; YO = yolk sac tumor; CH = choriocarcinoma)

Table 3. Histological composition of combined tumors

Histology	n
SE + EC	3
SE + EC + T	2
SE + EC + T + YO	4
SE + EC + T + YO + CH	1
SE + EC + YO	1
SE + T	3
SE + T + YO	1
SE + Scar#	

(SE = seminoma; EC = embryonal carcinoma; T = mature and immature teratoma; YO = yolk sac tumor; CH = choriocarcinoma)
#Tumor classified as combined tumor on the basis of nonseminomatous metastases.

The nonseminomas (n=32) were recent cases from the Surgical and Medical Oncology Departments. When it appeared that the material contained few patients with clinical stage I tumors at presentation, we added nine randomly picked cases (seven nonseminomas and two combined tumors) from our group of patients in stage I treated with orchidectomy alone (17). Fifteen seminomas were recent cases from the Radiotherapy Department. The material consisted predominantly of patients in clinical stage I at presentation. Therefore, five additional patients at higher stages were culled from our files to obtain a more balanced stage distribution. Inclusion of patients in the material was always decided upon before ploidy measurement. The only reason for not including measured cases was poor quality of DNA profiles: 3 nonseminoma and 2 combined tumor cases. The age of the patients is in accordance with the diagnosis. Noteworthy,

in keeping with the literature (6, 45), the age of the patients with combined tumors is in between that of seminoma and nonseminoma patients, and significantly different from both (Table 1).

Sampling for DNA flow cytometry

When possible, for every tumor a paraffin block or frozen tissue sample was selected for DNA flow cytometry, which contained all the different components present in that tumor. If not every component was present additional tissue was sampled so as to cover all the histological components. For orchidoblastomas, seminomas and pure nonseminomas usually one sample sufficed, for combined tumors one to two samples were needed. Most often the histological components were so intermingled that separation of the components by carving the block was not feasible. In the case of combined tumors it was most often possible to measure the seminoma and nonseminoma components separately, using one or two paraffin blocks or frozen tissue samples. Paraffin blocks were used in 48 cases (9 orchidoblastomas; 6 seminomas; 14 combined tumors; 19 nonseminomas), frozen tissue in 34 cases (1 orchidoblastoma; 14 seminomas; 2 combined tumors; 17 nonseminomas).

RESULTS AND DISCUSSION

Ploidy of testicular GCT

The distribution of the DNA index (DI) of all tumors is shown in separate scattergrams per histological type (Figure 1). The median DI of aneuploid stemlines of orchidoblastoma is significantly different from that of the other types. Seminoma has a median DI, which is significantly higher than that of both combined tumors and nonseminomas. The difference between combined tumors and nonseminoma is not significant. The median DI of the seminoma and nonseminoma components of the combined tumors is significantly different. On the other hand, the median DI of the seminoma component of combined tumors is not different from the median DI of pure seminomas. Similarly, the median DI of the nonseminoma component of combined tumors is not different from that of nonseminomas (Table 4). The median DI is not significantly influenced by type of tissue sample, clinical stage of the disease, or age of the patient (Table 4).

Four tumors had a (near)diploid main stemline: three orchidoblastomas, and one nonseminoma, consisting of mature teratoma only.

Multiple aneuploid stemlines were found in four orchidoblastomas,

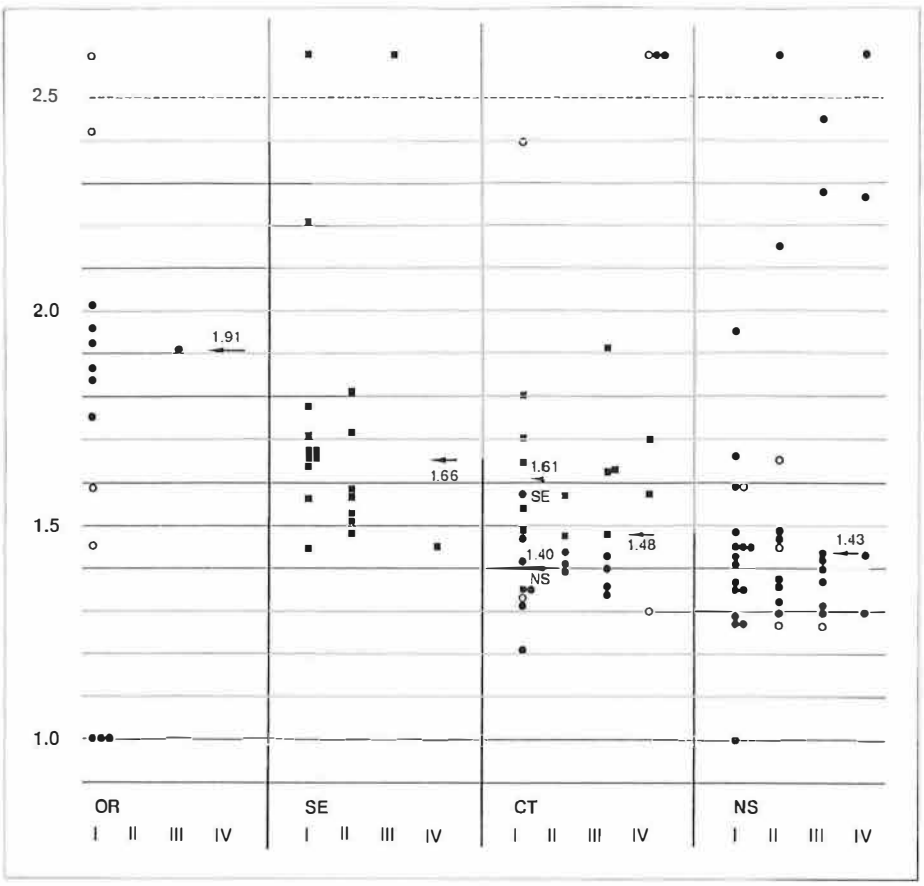

Figure 1. The DNA-index (DI) is shown for all testicular germ cell tumors, per tumor type. For orchidoblastoma (OR), nonseminoma (NS), and the nonseminoma component of combined tumors (CT) the DI of the main stemlines is indicated by a closed circle (o), the DI of additional stemlines by an open circle (o). The DI of the main stemlines of seminoma (SE) and the seminoma component of combined tumors is indicated by a closed square (▮).

The data are plotted in separate lanes for clinical stage I through IV tumors. The median DI of the main stemlines indicated by arrows, is for orchidoblastoma, seminoma, combined tumors, and nonseminomas respectively: 1.91, 1.66, 1.48, and 1.43. The median DI of the main stemlines for the seminoma and nonseminoma components of the combined tumors, indicated by arrow heads is respectively 1.61 and 1.40. The results of statistical analysis are shown in Table 4.

combined tumors.

Table 4. Median DNA index (DI) in histological types of germ cell tumors of the testis

Histological type/component	Median DI of main stemlines						
	all tumors/ components	paraff. tissue	frozen tissue	stage I	stage >I	age mean	age >mean
orchidoblastoma	1.91 #	NA*	NA	NA	NA	1.95	1.86
seminoma	1.66 #	1.72	1.64	1.67	1.57	1.67	1.66
combined tumor	1.48						
seminoma comp.	1.61 NS #	NA	NA	1.59	1.61	1.57	1.64
non-sem. comp.	1.40	NA	NA	1.38	1.40	1.40	1.40
non-seminoma	1.43	1.36	1.45	1.43	1.43	1.43	1.40

* NA, not applicable in view of uneven distribution over the two alternatives.
# $p<0.01$, unidirectional Mann Whitney U test; NS, not significant.

Neither the seminomas, nor the seminoma components of combined tumors had multiple aneuploid stemlines. Multiple aneuploid stemlines were found in the majority of the combined tumors. Out of 13 tumors in which both components could be measured separately, 10 showed clearly different stemlines in the seminoma and nonseminoma components. In eight of these cases the DI of the seminoma component was higher than that of the nonseminoma component (Table 5). In the tumors 7 and 12, where the nonseminoma component had the higher DI, it was about twice the median DI of nonseminoma, suggestive of a doubling of the DI as a result of polyploidization (Table 5). Without the latter event the nonseminoma component would have had the lower DI of the two components.

In our material diploid tumors appear to be rare among the GCTs of the testis of adults: only one case out of 72. These results are not in agreement with recent papers on DNA-flow cytometry of GCTs of the testis in adults, reporting 16% (52) and 36% (46) diploid tumors. An explanation might be that embryonal carcinoma cells are particularly fragile, and tend to be underrepresented as compared to normal host cells in suspensions prepared from paraffin embedded and frozen tissues. However, our results are consistent with earlier flow-cytometrical studies (14, 63) and with a study by Atkin and Kay (4) who used micro-densitometry to measure DNA-ploidy and found few (near)diploid tumors. Results of karyotyping of GCTs of the testis in adults also attest to the rarity of

diploid tumors (1, 2, 3, 10, 18, 19, 28, 37, 60). Clonally abnormal (near)diploid karyotypes are extremely rare. Even cases with the i(12p) marker chromosome as the sole structural abnormality have high modal chromosome numbers (18, and personal observation). Spermatocytic seminoma is the exception among the GCTs of adults: five out of 11 cases were diploid (32).

Table 5. DNA-index of separately measured seminoma (SE) and nonseminoma (non-SE) components of combined tumors.

Tumor no.	DI*	
	SE component	> = < non-SE component
1	-	- 1.36
2	1.63	> 1.43
3	1.64	- -
4	1.35	= 1.31
5	1.47	= 1.43
6	1.64	> 1.35
7	1.70	< 3.07
8	1.58	> 1.39
9	1.80	> 1.57
10	1.54	> 1.21
11	1.48	= 1.46
12	1.57	< 2.82
13	1.48	> 1.34
14	-	- 1.40
15	1.92	> 1.40
16	1.70	> 1.41

*8 x >; 3 x =; 2 x < (The DI of two components was considered different if the difference was 10%).

In contrast with the rarity of diploid tumors in our adult patient population, three out of ten infantile orchidoblastomas were (near)diploid.

We found a higher ploidy in seminomas than in nonseminomas. This was also demonstrated by Martineau by analysis of unbanded chromosomes (28), and by Quirke et al. using flow cytometry (46). On the other hand, Fossø et al. using DNA flow cytometry failed to demonstrate a difference in ploidy between seminoma and nonseminoma. However, even in their small number of tumors the median DI of seminomas is about 1.6 and of nonseminomas 1.4 (14), which is quite similar to our results.

Pathogenetic model

Recent data (35, 36, 49, 62) support the model of pathogenesis of seminomas and nonseminomas proposed by Ewing (12) and Friedman (15), in

which all GCTs (with the possible exception of spermatocytic seminoma (32)) pass through a seminoma stage as part of their development (8, for comment), as opposed to the model suggested by Pierce and Abell (43), in which seminomas and nonseminomas develop separately. Our data on ploidy of seminomas, combined tumors and nonseminomas, lend further support to the former model.

The results fit into a pathogenetic model shown in Figure 2. Carcinogenesis probably starts during intrauterine life (13, 53, 61). An early event might be polyploidization of a dysplastic germ cell precursor resulting in carcinoma in situ with a (near)tetraploid DNA content, i.e. a DI of about 2. The mean DI of carcinoma in situ cells was about two in eight infertile men who had not yet developed an invasive GCT (33).

Polyploidization as an early step in tumor progression followed by chromosome loss, is well documented for bladder and prostate cancer (55), and also demonstrated in an experimental tumor model (41). Tumor progression in testicular GCT is conceivably also the result of chromosomal events causing a net loss of DNA. Initially, loss of chromosomes results in invasive seminoma, a tumor of which the cells resemble gonocytes, as do carcinoma in situ cells (51). They have similar ultrastructural features (22), and both express placental alkaline phosphatase (24). Seminoma, being less aggressive than nonseminoma and probably earlier in the clonal evolution (34) will become clinically manifest at older age, with most patients in stage I (25).

Further loss of chromosomes results in a nonseminoma, a more advanced cancer in terms of tumor evolution, which has lost the capability of gonocytic differentiation. On average the tumor is more aggressive than seminoma, and thus becomes manifest at a younger age (Fig. 2) Only about 25-30% of the patients present without metastatic disease, and are thus curable by orchidectomy alone (40). The dedifferentiated tumor cell has the phenotype of an embryonal carcinoma cell, which may or may not be pluripotent. Depending on the lineage and degree of differentiation the nonseminoma may present as mixture of embryonal carcinoma with embryonic and extraembryonic tissues, as a pure tumor consisting of one of these tissues, or as a pure embryonal carcinoma, which may represent a nullipotent end-stage of tumor evolution. In our material the different components of mixed nonseminomas tend to have a similar DI as opposed to the seminoma and nonseminoma components in

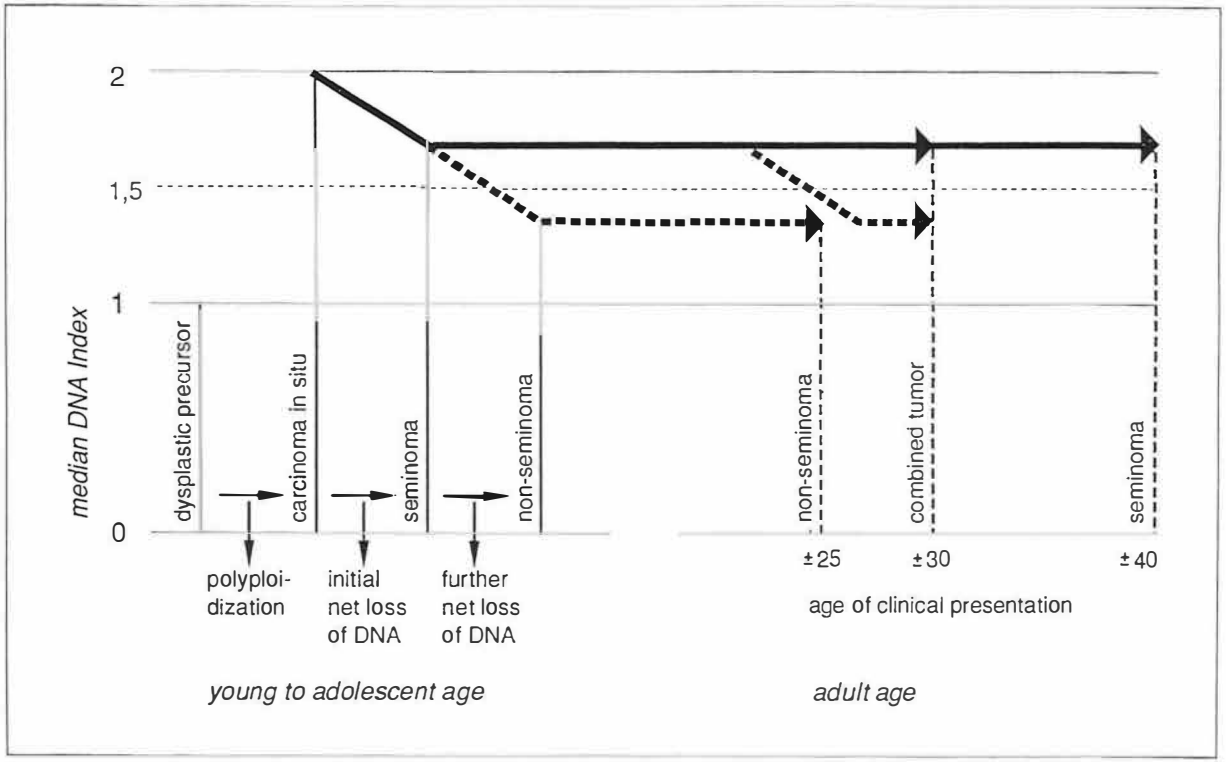

Figure 2. Schematic representation of a model of tumor progression in germ cell tumors of the adult testis. Supposedly, an early event is polyploidization of a dysplastic germ cell precursor, resulting in carcinoma in situ with a DI of about two. Initial net loss of DNA (chromosomes or parts of chromosomes endowed with tumor suppression properties), leads to invasive seminoma (solid line), a tumor type through which all other types progress. Rapid progression through the seminoma stage by further loss of DNA leads to nonseminoma (dotted line). Slower progression through the seminoma stage may result in combined tumor. Seminomas being the least aggressive, become clinically manifest at older age than the more aggressive nonseminomas. Combined tumors have an age of clinical presentation in between the two.

combined tumors. Probably the different components in mixed tumors are closely related, and differ by virtue of largely epigenetically determined direction of differentiation. The biologic behaviour of nonseminomas is not only determined by progression but to a large degree by the differentiation lineage. Angioinvasiveness of choriocarcinoma for example, is not the result of clonal tumor evolution per se, but rather inherent with trophoblastic differentiation (47). Differentiated teratomas of the testis, as another example, are malignant tumors, but of low malignant potential (7, 40), in keeping with their very high degree of somatic differentiation.

Patients with combined tumors present at an age in between that of seminoma and nonseminoma patients (6, 45). In our material the mean age of patients presenting with combined tumors is significantly higher than that of nonseminoma patients and significantly lower than that of patients presenting with a seminoma (Table 1 and Fig. 2). A striking finding is that the median DI of the seminoma and the nonseminoma components in combined tumors is similar to the DI of the pure seminoma and nonseminoma counterparts. In the majority of the cases in which the two components could be measured separately the seminoma component had the higher DI. This observation is compatible with the hypothesis that the nonseminoma component has evolved from the seminoma component through net loss of chromosomes.

The biologic behaviour of combined tumors is determined by the nonseminoma component, and they are treated accordingly. The fact that these tumors nevertheless appear at an older age as compared to nonseminomas may be due to the fact that they have a longer seminoma stage, thereby enabling the seminoma component to express itself. Our data support the contention that nonseminomas with a seminoma component are not just one variant of the mixed nonseminomas (31), but a separate entity (44).

In the only published case of a combined tumor in which both components were karyotyped using banding techniques, they had clonal abnormalities in common (5). Martineau, studying unbanded chromosomes, also found related karyotypes in the two components of combined tumors (28). These karyotypic data support the model in which nonseminoma progresses through a seminoma stage (Fig. 2).

Presently we are investigating whether the consistently lower DNA-ploidy of nonseminomas as compared to seminomas is due to non-random

loss of chromosomes. Preliminary results of a comparison of complete karyotypes of 13 seminomas and 15 nonseminomas, indicate that this is indeed the case. The involved chromosomes may harbor genes which play a prominent role in germ cell differentiation.

It is evidently more difficult to fit orchidoblastoma into the proposed model. Three out of ten tumors are diploid, apparently polyploidization is not obligate. When polyploidization has occurred, the DI of the tumors remains close to two (1.91), in keeping with a short period of tumor evolution in the young patients. The relatively low malignant potential of the tumors is reflected in the high proportion of patients presenting in stage I (27). The entirely different histological composition of testicular GCT of infants as compared to adults (20, 50) may be explained by epigenetic factors in the infantile testis. It could also be that the pathogenesis of testicular GCTs of infants is fundamentally different from those of adults, in view of the fact that carcinoma in situ could not be demonstrated in the vicinity of orchidoblastoma (26). On the other hand it is found in a very high proportion of testes containing GCT in adults (23, 26), with the exception of spermatocytic seminoma (32). Additional evidence for a different pathogenesis may be the occurrence of diploid tumors, and the absence of the i(12p) marker chromosome in the only orchidoblastoma that we have karyotyped. This marker is present in over 80% of testicular GCTs in adults, which we karyotyped (papers submitted).

Clinical relevance

The finding that primary testicular GCT of adults are so consistently aneuploid may be diagnostically useful in two situations. First, it may help in making the differential diagnosis between a primary testicular germ cell and non-germ cell tumor. When a tumor in a testis of an adult is diploid, it is probably not a GCT. In our limited experience non-germ cell tumors of the testis are usually diploid (two out of two lymphomas and two out of three Leydig cell tumors were diploid). In the second place it may help to differentiate between a primary extragonadal malignant GCT and a retroperitoneal or mediastinal metastasis from a primary testicular GCT. If a malignant GCT in the retroperitoneum or mediastinum is diploid, it is probably not a metastasis from a testicular GCT. Metastases from the latter are aneuploid like the primary tumors (37, 38, 63). On the other hand mediastinal malignant GCTs are diploid in a relatively high proportion of the cases:

six out of 12 in an unpublished series (and 39). Recently it was proposed that bilateral testicular biopsies be taken in patients with extragonadal malignant GCTs and clinically normal testes to exclude testicular origin of the tumor via demonstration of carcinoma in situ (9). Measurement of the DI of an extragonadal malignant GCT may be an additional, less invasive means to establish the extragonadal character of it.

In an increasing number of tumors aneuploidy has been shown to correlate with a poor prognosis (29 for review). Previous studies have failed to demonstrate such a relationship in GCTs of the testis (46, 52). Our results indicate that if one were to look for a prognostic relevance of ploidy in GCTs of the testis, one should not lump the different types together (52).

METHODS

DNA Flow Cytometry.

The method described by Hedley et al. (21) was applied for DNA flow cytometry on paraffin embedded tissue. Sections of 50 m thickness were cut and deparaffinated. Tumor tissue sections were digested using 0.5% pepsin (Sigma). Isolated cells were stained by suspending them in 1 g/ml 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Boehringer GmbH, Mannheim, West Germany) in RPMI 1640 tissue culture medium for 30 minutes at room temperature. From the same blocks 2-3 m thick sections were cut for routine HE slides, allowing a histologic check of the tumor components processed for DNA flow cytometry. Different components of the tumor tissue could be measured simultaneously or separately by engraving the paraffin blocks, so as to isolate the different components.

Fresh tumor tissue was processed using the methods developed by Vindeløv et al. (57). Tissue was stored in liquid nitrogen (59). Nuclei were isolated using a detergent-trypsin method and stained with propidium iodide (58). Flow cytometry was performed with the Ortho ICP 22 flow cytometer. Trout red blood cells (TRBC) were stained with propidium iodide and used as an internal reference for the frozen samples. From frozen tissue samples a cryostat section was made to histologically check the samples.

Evaluation of DNA profiles

Tumor ploidy was expressed by the DNA index (DI) defined as the ratio between the modal G0,1 peak of the aneuploid population to that of

the modal G0,1 peak of diploid normal cells in the samples. By definition a diploid tumor cell population thus has a DI = 1.00. In DNA profiles from frozen samples the diploid G0,1 peak could be identified on the basis of its relative position to the G0 peak of the TRBC ploidy reference (56, 57). In DNA profiles from paraffin-embedded material, the most left peak was considered to represent the diploid population (because no stable G0,1/TRBC ratio is obtainable under these circumstances (48)). A tumor was classified as aneuploid when the DNA profile showed two distinct G0,1 peaks and as multiploid when multiple aneuploid stemlines were present. When the DNA profile showed a shoulder, a single G0,1 peak with a high coefficient of variation (CV) or an ill-defined subpopulation the measurements were repeated. Tumors showing a single G0,1 peak with 5.5% < CV < 10% were classified as near-diploid. DNA profiles with CV`s over 10% were considered uninterpretable.

Statistical analysis

The results were statistically analyzed using a unidirectional Mann-Whitney U test (11).

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CHAPTER III

CYTOGENETICAL ANALYSIS OF TEN SEMINOMAS, TWO OF THEM LACKING THE i(12p)

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ABSTRACT

A cytogenetic analysis of ten seminomas has been carried out after direct harvesting of the tumor cells. Modal chromosome numbers ranged from 63 to 112, in agreement with flow cytometric determination of the DNA content of the tumors. Eight tumors had an i(12p) among other chromosomal abnormalities. Two seminomas lacked the i(12p). Testicular germ cell tumors lacking the i(12p) may represent a separate group of germ cell tumors.

INTRODUCTION

Most chromosome studies of malignant germ cell tumors of the testis have been carried out in primary nonseminomas, and in cell lines derived from primary or metastatic testicular germ cell tumors. We are aware of only a few descriptions of the chromosomal constitution of seminomas [1-8]. A full cytogenetic description of three seminomas [6] and a partial description of ten [5] are included in these.

Further cytogenetic studies of seminomas will probably give some insight in the pathogenesis, oncogenesis, and progression of this type of malignant germ cell tumors of the testis, as well as in the possible relationship between seminomas and nonseminomas. We report the results of a cytogenetic analysis and DNA flow cytometry of ten seminomas of the classical type and compare our data with the few that have been published previously.

MATERIALS AND METHODS

Direct harvesting of tumor cells for karyotyping [7], and measurement of cellular DNA content by flow cytometry [9] were carried out on morphologically checked, representative, fresh samples of all tumors. Additionally, the nonseminoma component of Case 6 (combined tumor) was harvested after short time tissue culture.

From paraffin-embedded tissue of the tumors three additional samples were taken for measurement of cellular DNA content [9]. DNA content is expressed as a DNA index (DI), i.e. the ratio of the tumor and the normal G1 cells (a diploid cell has a DI = 1).

In what concerns statistical processing of the chromosomal findings, the number of copies per chromosome was analyzed for ten cases with 2 way analysis of variance.

RESULTS

Data concerning patient age, clinical stage, histology, DNA index, number of abnormal metaphases analyzed, and modal chromosomal counts are summarized in Table 1.

Table 1. Summary of the clinical and cytogenetic data

CASE	PATIENT AGE (yrs)	CLINICAL STAGE	HISTOLOGY	DNA INDEX	No. OF CELLS ANALYZED	MODAL NUMBER
1	35	II-C	Classical seminoma	2.62	3	112
2	34	I	Classical seminoma	1.53	3	68
3	43	I	Classical seminoma	2.38	8	109
4	31	I	Classical seminoma	2.5	10	106
5	48	I	Classical seminoma	1.6	10	71
6	32	I	Combined tumor#	1.48*	10**	72
7	43	I	Classical seminoma	1.58	7	71
8	48	I	Classical seminoma	1.57	5	65
9	26	II-C	Classical seminoma	1.42/1.51(a)	7	63
10	58	I	Classical seminoma	1.69	2	7

Classical seminoma/embryonal carcinoma/differentiated and undifferentiated teratoma/yolk sac tumor.
* DI of the seminoma component. ** No metaphases were obtained from the nonseminoma component.
(a) Both stemlines were present in the DNA flow graph.

KARYOTYPES

A representative karyotype of each case is described in Table 2. Figures 1, 2, 3, and 4 show karyotypes of representative metaphases of, respectively, cases 1, 4, 5, and 6. The numerical abnormalities can be deduced from Table 3.

Table 2. Karyotypical description of representative metaphases of each case

Case 1 - 117,XY,+X,+Y,+1,+2,+2,+4,+4,+5,+6,+6,+6,+7,+7,+7,+8,+8,+9,+9,
+10,+10,+10,+10,+11,+12,+12,+12,+12,+12,+14,+14,+14,+14,+15,
+15,+16,+16,+16,+17,+18,+18,+19,+19,+19,+20,+20,+20,+21,+21,
+21,+22,+22,+22,+der(X)t(X;?)(q28;?),+der(1)t(1;15)(p11;q11),
+del(1)(p11),+der(1)t(1;?)(p22;?),+der(1)t(1;?)(q11;?),
+del(1)(q11),+der(2)t(2;5)(q37;p15),+del(3)(q24),
+dic der(3)t(3;12)(3qter-->p25::12q24-->pter),
+der(7)t(5;7)(q13;q36),+der(7)t(5;7)(q13;q36),+i(12p),+i(12p),
+i(12p),+i(12p),+der(14)t(14;?)(q24;?),+M1,+M2,+M3(der(7)).

Case 2 - 69,XY,+X,+2,+3,+4,+6,+7,+7,+8,-9,+10,+12,+14,+15,+20,+20,+21,
+21,+21,+22,+del(1)(q31),+i(12p),+i(12p),
+dic der(16)t(2;16)(2pter-->q33::16p13-->qter),
+der(20)t(9;20)(20qter-->p12::9p23-->qter),
+der(22)t(22;?)(q13;?).

Case 3 - 104,XY,+X,+X,+X,+Y,+1,+1,+2,+3,+3,+3,+4,+5,+6,+6,+7,+7,+8,+8,
+8,+9,+10,+12,+12,+12,-13,+14,+14,+15,+15,+15,+15,+16,+16,+16,
+18,+19,+19,+20,+20,+21,+21,+21,+22,+22,+22,+22,+22,+22,+i(Xp),
+i(Xp),+der(1)t(1;?)(q32;?),+i(1q),+i(2q),+der(7)t(7;?)(q22;?),
+i(8q),+der(12)t(12;?)(p12;?),+i(12p),+M1,+M2,+M3.

Case 4 - 106,X,-Y,+X,+X,+1,+1,+2,+2,+2,+3,+4,+4,+5,+6,+6,+6,+7,+7,+7,+7,
+8,+8,+8,+8,+9,+9,+9,+10,+11,+12,+12,+12,+13,+14,+14,+15,+15,

+15,+16,+16,+17,+18,+18,+19,+19,+20,+20,+20,+21,+21,+21,+21,
+21,+22,+22,+22,+22,+der(1)t(1;?)(p11;?),+i(2q),
+der(3)t(3;?)(p23;?),+del(12)(q24.2),+der(15)t(15;?)(q22;?),
+der(15)t(15;?)(q22;?).

Case 5 - 71,XY,+X,+Y,+1,+2,+3,+3,+6,+7,+7,+8,+8,+8,+10,-11,+12,+14,+15,
+15,-17,-18,+19,+20,+der(9)t(9;?)(q13;?),
+der(11)t(1;11)(q21;q22),+der(11)t(11;?)(q11;?),
+der(11)t(11;?)(p11;?),+der(12)t(12;?)(q24;?),+i(12p),
+der(16)t(16;?)(q24;?),+i(17q),+M1.

Case 6 - 71,XY,+X,+Y,+1,+2,+3,+7,+7,+8,+8,+9,+12,+14,+14,+15,+16,-18,
+19,+20,+21,+21,+21,+22,+der(12)t(12;?)(p12;?),
+der(12)t(12;?)(q11;?),+i(12p),+i(12p),+i(12p).

Case 7 - 72,XY,+X,+1,+2,+3,+6,+7,+7,+8,+8,+9,+10,+12,+14,+15,+15,+16,
+17,+19,+20,+21,+21,+22,+22,+22,+der(4)t(1;4)(q21;q34),
+der(12)t(12;?)(p12;?).

Case 8 - 64,XY,-1,+6,+7,+10,-11,+12,+12,-13,-17,-18,+19,+20,-22,
+der(X)t(X;?)(p21;?),+der(1)t(1;?)(p13;?),+der(1)t(1;?)(p11;?),
+der(7)t(7;?)(q22;?),+dup(11)(q13-->23),+dup(11)(q13-->23),
+der(12)t(12;15)(p11;q11),+i(12p),+der(13)t(13;14)(p11;p11),
+der(17)t(17;?)(q25;?),+der(19)t(19;?)(q13.4;?),
+der(20)t(20;?)(p12;?),+der(21)t(21;?)(p11;?),
+der(22)t(1;22)(p11;p11),+M1,+M2,+M3.

Case 9 - 63,XY,+X,+1,+2,-3,+6,+7,+7,+8,+8,-11,+12,-13,+15,+15,+17,+19,
+20,+21,+21,+22,+der(3)t(3;?)(p21;?),+der(11)t(11;14)(p13;q13),
+i(12p).

Case 10- 73,XY,+X,+1,+2,+2,+3,-4,+8,+9,-11,-13,+14,+15,+15,+16,+17,+17,
-18,+19,+19,-21,+der(1)t(1;?)(p11;?),+del(3)(q11)*,
+der(4)t(4;?)(q35;?),+der(4)t(4;7)(q21;q11.1),
+der(7)t(4;7)(q12;p15),+der(7)t(7;?)(q21;?),+i(8q),
+der(8)t(8;?)(q11;?),+der(9)t(9;?)(q12;?),
+der(11)t(11;?)(q23;?),+der(12)t(5;12)(q33;q24),+i(12p),
+der(21)t(21;?)(p11;?)*,+M1,+M2*,+M3*,+M4*.

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* Only present in one metaphase.

STATISTICAL ANALYSIS

The summary table for the analysis of variance is shown below:

EFFECT	SSQUARES	OF	MS	F	P
Chromosomes	124.3	23	5.4	7.0	<0.001
Cases	134.0	9	14.9	19.35	<0.001
Error	160.4	207	0.77		
Total	418.7	239			

All effects are highly significant, indicating that chromosomes are present in different numbers and that persons have different total numbers of chromosomes. The interaction term, which is used as error term, is numerically rather small, indicating that most of the variability is explained as a combination of differences per person and differences per chromosome.

Table 3. Modal number of normal chromosomes and i(12p) per case

	MODAL NUMBER OF NORMAL COPIES OF CHROMOSOMES PER CASE																								No. Of
CASE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	i(12p)
1	3	4	2	4	3	5	5	4	4	6	3	7	2	6	4	5	3	4	5	5	5	5	2	2	4
2	2	3	3	3	2	3	4	3	1	3	2	3	2	3	3	2	2	2	2	4	5	3	2	1	2
3	4	3	5	3	3	4	4	5	3	3	2	5	1	4	6	5	2	3	4	4	5	7	4	2	1-2
4	4	5	3	4	3	5	6	6	5	3	3	5	3	4	5	4	3	4	4	5	7	6	3	0	0
5	3	3	4	2	2	3	4	5	2	3	1	3	2	3	4	2	1	1	3	3	2	2	2	2	1
6	3	3	3	2	2	2	4	4	3	2	2	3	2	4	3	3	2	1	3	3	5	3	2	2	3
7	3	2	3	2	2	3	4	4	3	3	2	3	2	3	4	3	3	2	3	3	4	5	2	1	0
8	1	2	2	2	2	3	3	2	2	3	1	4	1	2	2	2	2	1	3	3	2	1	1	1	1
9	3	3	1	2	2	3	4	4	2	2	1	3	1	2	4	2	3	2	3	3	4	3	2	1	1
10	3	4	3	1	2	2	2	3	3	2	1	2	1	3	4	3	4	1	4	2	1	2	2	1	1

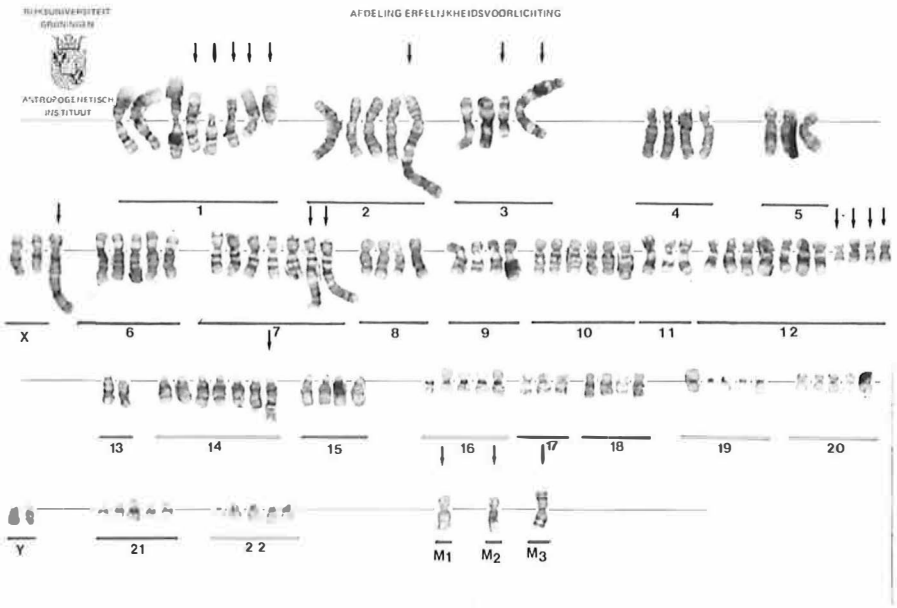

Figure 1 - Karyotype of a representative metaphase of Case 1 (the karyotypical description is given in Table 2).

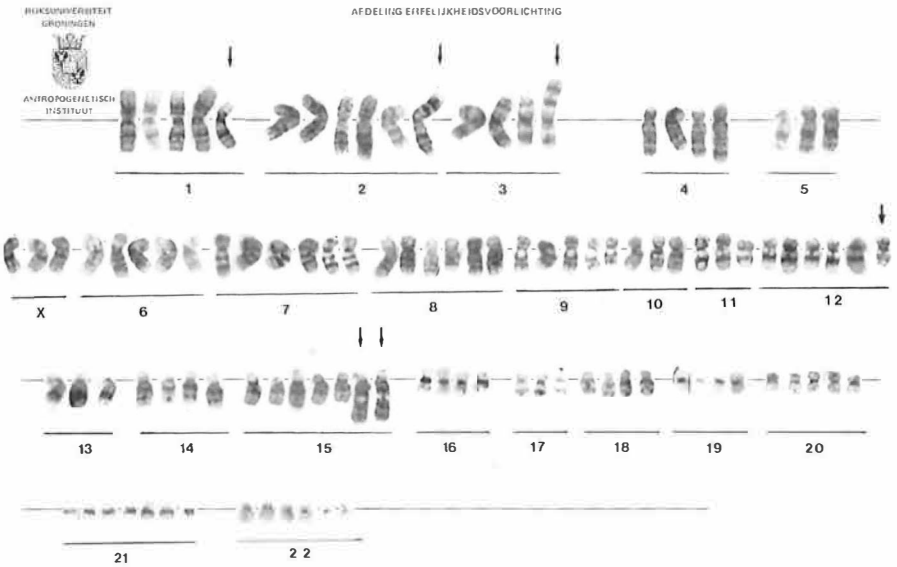

Figure 2 - Karyotype of a representative metaphase of Case 4 (the karyotypical description is given in Table 2).

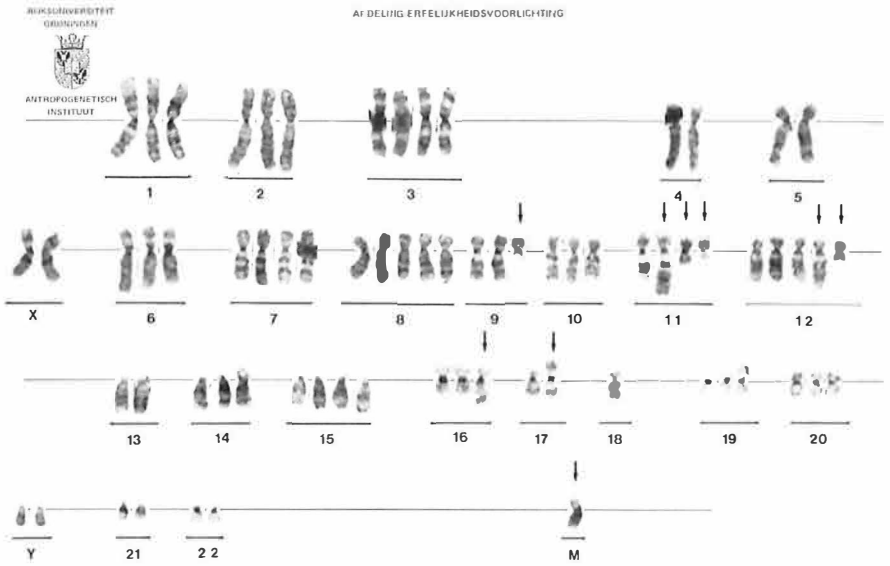

Figure 3 - Karyotype of a representative metaphase of Case 5 (the karyotypical description is given in Table 2).

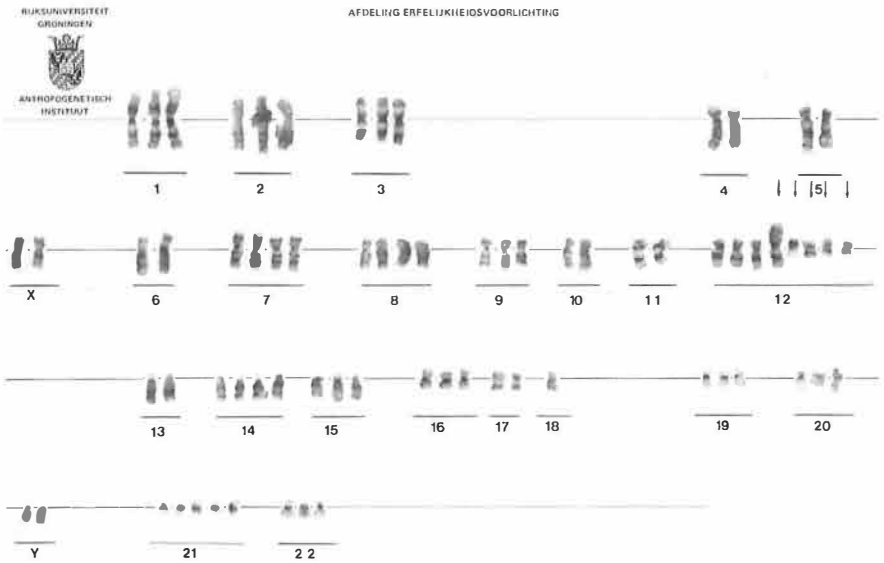

Figure 4 - Karyotype of a representative metaphase of Case 6 (the karyotypical description is given in Table 2).

To indicate the effect of the relative numbers of chromosomes, Figure 5 shows the chromosome counts combined for all cases, after giving each person the same weight, irrespective of his total chromosome count, which is arbitrarily set to 46 in this calculation.

Multiple comparison using the Newman-Keuls method, shows that chromosome 13, 11 and 18 are less frequently found than chromosomes 21, 15, 8, 7 and X.

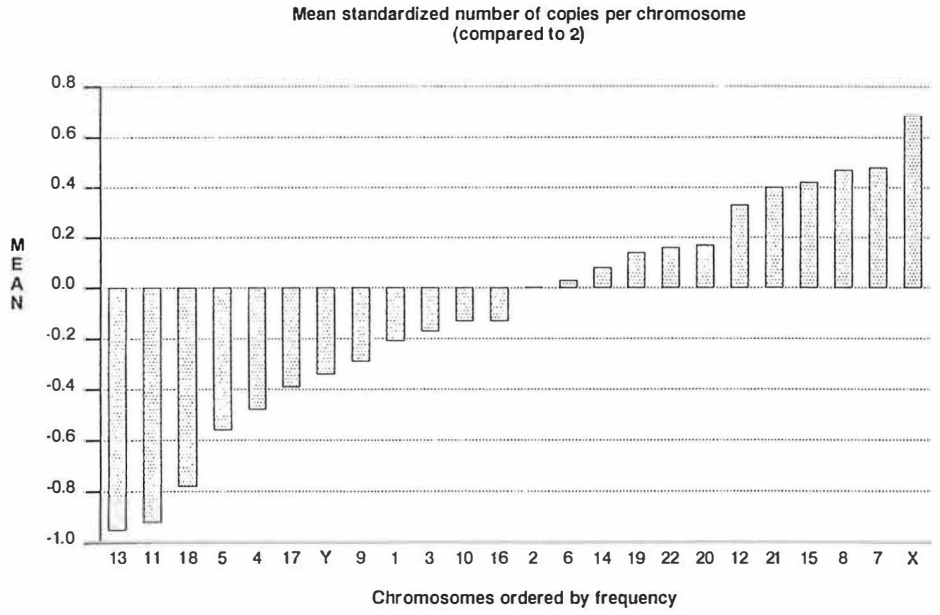

Figure 5 - Average of the standardized number of normal copies of chromosomes per case (compared to 2). Every case was given equal weight in terms of chromosomal counts, which was set arbitrarily to 46.

DISCUSSION

NUMERICAL ABNORMALITIES

Early cytogenetic studies of testicular germ cell tumors [1-4,10] described a generally hyperdiploid to hypotriploid chromosome complement with higher modal chromosome counts in seminomas than in non-seminomas. Recent studies [5,8-12] confirm the early observations about the difference in chromosome numbers according to the histology of these tumors. Table 4 shows that there is a considerable variation in the average of modal chromosomal counts reported by different authors, higher averages being clearly associated with larger series. This

finding is probably due to the higher probability that larger series will also include less some less frequently occurring seminomas with unusual high chromosome counts.

Table 4. Summary of the published data on chromosomal counts in seminomas

AUTHORS	No. OF TUMORS	MODAL CHROMOSOME NUMBERS OR RANGES	AVERAGE OF MODES
Fisher & Golob [2]	1	54-56	55
Rigby [16]	3	61,68,77	69
Atkin & Baker [5,13]	11	60-63,80-81,73,73-77,72,95, 69,102-105,55-61,92,99	80
Martineau [3,15,(a)]	11	64,67,69,74,78,84,87,94,156, 76-80,87-94	86
Lelikova et al. [14]	18	60-61,60-64,61-62,65 and 67, 66-67,66-68,67-69,69,69,77 and 80,69-71,83-85,84-101, 90,91-93,107-108,137,115-138	82
PRESENT STUDY	10	63,65,68,71,72,73,73,104,109, 112	85

(a) Martineau M. Unpublished data (quoted by Atkin NB [4]).

Plotting the number of seminomas against their modal range (Figure 6), it seems clear that there is a preferential clustering of cases around multiples of 23 (starting with 69).

Several hypotheses have been proposed on the mechanism of origination of aneuploidy in seminomas: successive non-disjunctions of a diploid cell, polyploidization and cell fusion, followed and/or preceded by chromosomal gain and/or loss [see 17 for review]. Since there are almost no reported cases of seminomas with less than 60 chromosomes, it seems rather unlikely that successive random non-disjunctions of a diploid cell could be a mechanism of oncogenesis of seminomas. If that were to be the case, one would expect a higher frequency of near diploid seminomas and lower frequencies in higher classes. Since the opposite is observed, there should be other modes of origin of aneuploidy.

The clear peak in the triploid range may better be explained by loss of chromosomes starting in a tetraploid cell. This would be in keeping with Müller's [18] finding of a DI of 2 in testicular carcinoma in situ (CIS) cells.

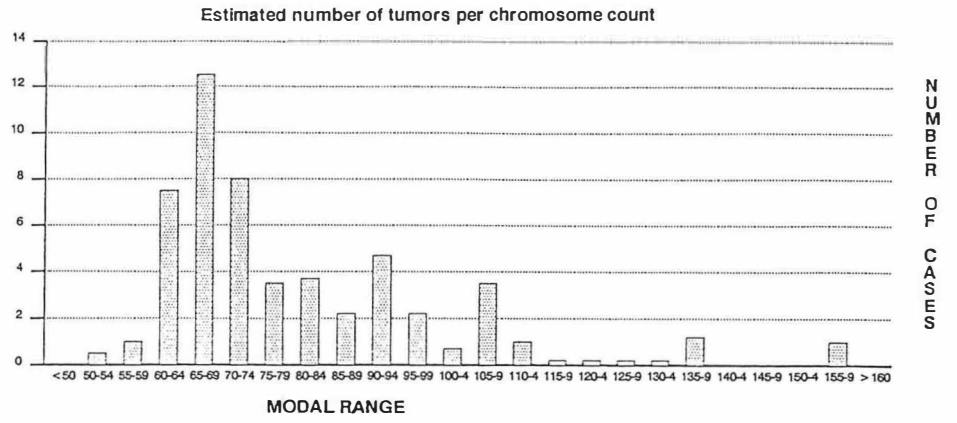

Figure 6 - Estimated number of published primary seminomas per chromosome count. When the reported modal counts of a case did not fit in a single class in the graph, the case was plotted as equal fractions in the corresponding classes.

However, in adults presenting with invasive testicular cancer, we have found several times a DI around 1.5 in the adjacent CIS compartment[1]. This finding may either represent a parallel evolution of the tetraploid CIS cell and the (presumably) derived invasive cancer cells, or alternatively the existence of triploid CIS cells ab initio. Therefore, we cannot exclude the possibility that the clustering of seminomas around triploid counts may be a characteristic of testicular germ cell tumors of adults, related in some way to meiosis. Conceivably, either fusion between a haploid post-meiotic cell with a diploid cell or a meiotic error leading to triploidization may be initially involved in the oncogenesis of seminomas.

Since the first cytogenetical studies of seminomas it has been noticed that there was an apparent nonrandom gain and/or loss of certain chromosomes (see [17] for review), which we could also confirm in our sample. Tables 2 and 3 give an idea of which chromosomes (or parts of chromosomes) are usually over and/or underrepresented. As can be seen, #7, #8, #12 (mainly because of its short arm), #15, #21 and X are overrepresented, while #11, #13, #17 and #18 are underrepresented.

Although the precise meaning of these findings in seminomas remains to be explained, it seems reasonable to consider the following pos-

[1] Oosterhuis JW. Unpublished data.

sibilities:

- Chromosomes (or chromosomal regions) consistently underrepresented may contain tumor suppressor genes, which by elimination would give raise to malignancy, in a similar way to what happens in retinoblastoma and Wilms's tumor [19-21] and to what has recently been found in some nonhereditary cancers [22,23].

The suggestion from cell hybrids studies that #11 [24-27] and #13 [23,24] are probably important for general tumorsuppression seems therefore in keeping with their consistent underrepresentation in seminomas.

Events leading to loss of heterozygosity probably play a crucial role in oncogenesis [27-32]. Unfortunately, the few published cytogenetic and enzymatic studies on loss of heterozygosity for regions in #1p and #13p in testicular cancers [33-36] have been based on cell lines. Since chromosomal abnormalities can arise during culture, it is difficult to extrapolate the conclusions obtained from a study of cell lines to the in vivo situation. Besides, we are not aware of similar studies specifically in seminomas, probably due to difficulties in keeping them in culture [3,8].

Further DNA studies using either fresh tumor material, xenografted tumors or tumor cells after a short term culture will be necessary to confirm the conclusions derived from cell line studies.

- Overrepresented chromosomes (or part of chromosomes) may contain genes causing some growth advantage.

Since tumor suppression seems to be "doses dependent" [28-31], sometimes showing a kind of titration effect [30], malignancy would then be the result of an unbalance between tumor suppressing and tumor promoting genes, in favor of the latter.

In all studied cases we found that chromosome counts of abnormal metaphases within each tumor were very homogeneous, which is in agreement with previous reports of Martineau [3] and Atkin [4], although at variance with the findings of Delozier-Blanchet [8]. In our experience, DNA flow cytometric graphs of seminomas are more homogeneous than in nonseminomas, which lends further support in our cytogenetic findings.

STRUCTURAL ABNORMALITIES

As can be seen in Table 2, we found that in seminomas #12 was the chromosome most often involved in structural abnormalities in seminomas.

Eight out of ten seminomas had in common one or more copies of i(12p) a specific marker for germ cell tumors of the testis and, possibly, of the ovary [37,38]. Two seminomas lacked this isochromosome. The two "i(12p) negative seminomas" (cases 4 and 7), however, contained structural abnormalities of chromosome 12, involving bands 12p12 and 12q24, respectively. Although these bands contain the loci for k-ras 2 [39], and a sequence homologous to k-ras 2 [40], respectively, only molecular studies can tell whether k-ras 2 and its related sequence are in some way involved in the chromosomal abnormalities of cases 4 and 7. In former reports [5,7,8] there was always at least one copy of i(12p), and we are not aware of any previous reports of banded cytogenetical studies of seminomas that lack this marker. Delozier-Blanchet [8] found the i(12p) in duplicate or triplicate only in non-seminomatous germ cell tumors of the testis, which is at variance with Atkin and Baker's [5] and our findings. It is likely that i(12p) plays a decisive role in the oncogenesis of testicular germ cell tumors. If this is so, "i(12p)-negative seminomas" may either represent a different group of germ cell tumors, where malignancy is related to other events, or in both groups there may be a common underlying molecular event leading to malignancy. We could not find any clinical or histological criterion to discriminate between "i(12p)-positive and negative seminomas". Clinical prospective studies will be necessary to detect or rule out any possible differences between the two groups.

Besides i(12p) the only structural abnormalities common to different seminomas were other isochromosomes (Table 2). This finding may reflect the importance of genes located in the duplicated segment for tumor progression. We found an i(17q) in 2 seminomas (one not included in this series), as did Atkin and Baker [5]. An isochromosome for the long arm of chromosome 17 is a common abnormality in hematologic malignancies and has also been found in several solid tumors [41]. This isochromosome may thus represent another nonrandom (although not site specific) abnormality in seminomas and besides give further support to the hypothetical relationship between germ cell and hematological malignancies [43-46].

We have also observed a very frequent involvement of #1 in structural abnormalities, which is in agreement with the findings of Atkin and Baker [7]. Chromosome 1 rearrangements, however, are very common, also in non-seminomatous germ cell tumors of the testis

[7-9,11,33-36,42], as well as in hematologic malignancies and other solid tumors [41]. We could find no support in our series for the possible relationship of structural abnormalities of #1 and enhanced metastatic potential suggested by Delozier Blanchet [8].

The fact that no structural abnormalities of #6, #10, and #18 were found in our series (Table 2) nor in the cases reported by Atkin and Baker [7], may be either coincidental or a suggestion that those chromosomes contain genes whose balanced expression may be essential for cell survival in this environment. Alternatively, there may have been duplication after loss of the homologous chromosome, in which case the event could be critical for the oncogenesis and/or progression of seminomas.

ACKNOWLEDGEMENTS:

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CHAPTER IV

CHROMOSOMAL CHANGES IN PRIMARY TESTICULAR NONSEMINOMAS

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ABSTRACT

A cytogenetical analysis of 14 primary testicular nonseminomas has been carried out after short term tissue culture. The modal chromosome numbers ranged from 53 to 113, in agreement with the flow cytometric determination of the DNA content of the tumors. An apparent nonrandom excess of normal copies of chromosomes #8, #12 and X and deficiency of #9, #10, #13, #15, #18, #19 and Y was noted. At least one copy of an i(12p) was present in 12 tumors. 2 tumors, however, lacked that marker.

The chromosomal findings in primary nonseminomas and seminomas are compared.

INTRODUCTION

Testicular germ cell tumors of adults can be divided both clinically and morphologically in two distinct entities, seminoma and nonseminoma [1-3]. The latter may have one or more of the following histological subtypes: embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma. In the British classification [4] tumors with a seminoma and a nonseminoma component are classified as combined tumors, in the WHO classification as nonseminomas [3]. In about 20% of germ cell tumors seminoma and nonseminoma coexist [4,5]. In general, seminomas are less aggressive than nonseminomas, although the aggressiveness of the latter depends on the histological subtype, in particular the presence of embryonal carcinoma, yolk sac tumor, and/or choriocarcinoma.

From the different theories on the pathogenetic relationship between seminomas and nonseminomas [2,6-13] two main concepts emerge. One model updates of the former hypotheses of Pierce and Abell [8] and assumes that seminomas and nonseminomas independently derive from transformed (dysplastic) intratubular germ cells via carcinoma in situ [2,10,11]. Another model suggests that all testicular germ cell tumors (with the possible exception of spermatocytic seminoma) have a single origin in carcinoma in situ and progress through a seminoma stage [9,12,13]. This hypothesis represents a further development of the theories of Ewing [6] and Friedman [7].

Cytogenetic studies of seminomas and nonseminomas, as well as of the seminoma and nonseminoma components of combined germ cell tumors of the testis, may clarify the possible relationship between the different subtypes of testicular germ cell tumors.

Recently, we presented our cytogenetic findings in seminomas[1] and in a combined germ cell tumor of the testis[2].

Now we report the results of the cytogenetic analysis and DNA flow cytometry of fourteen primary nonseminomas.

MATERIALS AND METHODS

The tumors were submitted fresh and sterile and were processed for tissue culture and DNA flow cytometry, basically as described [14]. For chromosome preparations the tumor cells were harvested either by brief trypsinization or according to the procedures of Gibas et al. [15]. Colcemid (0.05-0.5 g/ml culture medium) was added two to five hours before harvesting. After harvesting, the cells were centrifuged for 5 minutes at 240 g. The pellets from the trypsinized cells were resuspended in 0.06 M KCL, incubated at 37°C for 15 minutes, centrifuged, resuspended in a mixture of methanol/acetic acid (3:1), centrifuged, resuspended, and left in the tubes for 20 minutes. The pellets from the cells harvested as described by Gibas et al. were immediately resuspended in fixative. In both methods there was a final centrifugation, after which the cells were resuspended and pipetted onto slides. Air dried chromosome preparations were GAG and/or GTG banded. In addition, tumor cells from cases 11 and 12 were directly harvested for chromosomal study [16].

For a statistical evaluation of the chromosomal findings, the number of normal copies per chromosome was analyzed for 14 cases with a two way analysis of variance.

RESULTS

A summary of the clinical, histopathological, and cytogenetical data of all cases is given in Table 1.

KARYOTYPES

A representative karyotype of each case is described in Table 2.

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[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Te Meerman G, Dam A, Koops HS. Cytogenetical analysis of ten seminomas, two of them lacking the i(12p). (submitted)

[2] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Buist J, Koops HS. Cytogenetical study of a combined germ cell tumor of the testis. (submitted)

The numerical abnormalities can be deduced from Table 3. Figures 1-4 show representative karyotypes of, respectively, cases 3, 9, 10, and 14. Cases 2 and 11, case 7, and case 12 have been described previously [1,17,2].

Table 1. Summary of the clinical and cytogenetical data of the 14 cases

CASE	PATIENT AGE (yrs)	CLINICAL STAGE	HISTOLOGY	DNA INDEX	No. OF CELLS ANALYZED#	MODAL NUMBER
1	19	I	EC/TI/TD/CHO	1.45	12	65
2	24	I	EC/TI/TD	1.27	9	55
3	19	IV	EC/YS	2.27 (1.26)	7	57
4	24	I	TD	1.45	15	65
5	19	I	EC/TD/TI	1.59 (1.42)	7	64
6	22	I	EC/TD/YS	1.27	5	59
7	23	IV	EC/TI/TD/YS	2.26	24	102
8	23	I	TI/TD	1.28	9	57
9	?	II-C	EC	1.28	9	59
10	23	I	EC/TD	1.26	10	60
11	64	II-C	SE/EC/TD/TI	1.33 (1.68*)	9	53
12	24	E	SE/EC	2 (2.51*)	7	101
13	24	IV	EC/TI/TD/YS	NM	11	62
14	31	I	EC/TI/TD/YS	2.43 (1.47**)	13	113

only abnormal metaphases. SE- seminoma; EC- embryonal carcinoma; TI- immature teratoma; TD- differentiated teratoma; YS- yolk sac tumor. * DI of the seminoma component.
** Secondary stemline. NM- not measured.

STATISTICAL ANALYSIS

The summary table for the analysis of variance is shown below:

EFFECT	SSQUARES	DF	MS	F	P
Chromosomes	113.1	23	5.0	8.4	<0.001
Cases	190.8	13	14.7	25.00	<0.001
Error	175.4	299	0.59		
Total	479.3	335			

All effects are highly significant, indicating that normal copies of chromosomes are present in different numbers and that persons have different total numbers of chromosomes. The interaction term, which is used as error term, is numerically rather small, indicating that most of the variability is explained as a combination of differences per person and differences per chromosome.

1 Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Buist J, Sleijfer DTh. "i(12p) negative germ cell tumors. A different group? (submitted)

2 Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Buist J, Koops HS. Cytogenetical study of a combined germ cell tumor of the testis. (submitted)

Table 2. Karyotypical description of a representative metaphase from each case

Case 1 - 66,XY,+X,+1,+4,+7,+7,+8,+8,+12,+12,-13,+15,+15,+17,+20,+21,
+21,+der(6)t(6;?)(p25;?),+i(12p),+i(12p),+M1(1qter-->q11::?),
+M2,+M3.

Case 2 - 55,XY,+6,+7,+7,-11,+12,+der(2)t(1;2)(p32;q35),
+der(3)t(3;4?)(p11;p11?),+der(8)t(8;?)(q24;?),
+der(11)t(11;?)(q25;?),+der(17)t(17;?)(p11;?),
+der(21)t(1;21)(q12;p11).

Case 3 - 57,XY,+X,+1,+2,+7,+12,-14,-20,+21,+del(7)(q32),+i(12p),+i(12p),
+der(13)t(13;14)(13qter-->p11::14q11-->q31),+del(17)(p11),
+M1,+M2(del(20)(q11)?).

Case 4 - 64,XY,+X,+X,+2,+3,+4,+6,+7,+8,-11,+12,+14,+16,+17,+21,
+der(1)t(1;11)(p34;q13),+der(11)t(1;11)(q12;q23),+i(12p),
+i(12p),+i(12p),+M(der(7)?).

Case 5 - 64,XY,+X,+X,+X,-1,+2,+3,+6,+7,+12,-14,-15,+16,+17,+18,+21,+21,
+22,+del(1)(p35),+del(1)(p35),+der(8)t(8;?)(q24;?),+i(12p),
+i(12p),+i(12p),+der(15)t(15;?)(p11;?).

Case 6 - 59,XY,+Y,+3,+6,+11,+12,+16,+17,+inv(X)(p11.2 p22.1),
+der(8)t(8;?)(q24;?),+i(12p),+i(12p),+i(12p),
+M(1qter-->q11::?).

Case 7 - 102,XY,+X,+X,+X,+Y,+1,+1,+2,+2,+3,+3,+3,+4,+4,+5,+6,+8,+8,+8,
+9,-11,+12,+12,+13,+13,+14,+14,+15,+15,+16,+17,+18,+18,+19,+20,
+20,+20,+20,+21,+21,-22,+der(1)t(1;?)(p13;?),+del(3)(p23),
+der(5)t(5;?)(q31:?),+del(6)(q21),+der(7)t(7;7)(p15;q11),
+inv(7)(p15p22),+del(8)(p12),+del(9)(p11),+del(10)(p13),
+der(11)t(11;14)(q14;q11),+der(11)t(11;?)(q25;?),
+del(12)(q15q24),+del(12)(q15q24),+i(12p),+i(12p),+i(12p),
+i(12p),+i(22q),+i(22q),+M1(der(7)?).

Case 8 - 59,XY,+X,+2,+6,+8,+12,+15,+16,+17,+20,+del(1)(p21),+i(12p),
+i(12p),+i(12p).
(also clonal: der(1)t(1;1)(p22;q21))

Case 9 - 59,XY,+X,+Y,+7,+8,-11,+12,+14,+20,+der(1)t(1;?)(p11;?),
+del(2)(q31),+i(12p),+i(12p),+i(12p),+der(14)t(14;?)(q24;?),
+der(17)t(17;?)(q25;?).
(also another clone with a del(1)(p34-->q42),and without the
der(1) and the der(14)).

Case 10- 60,X,-Y,+X,-2,+3,-4,+7,+8,+8,-11,+12,+14,+17,+20,+20,+21,+21,
+21,+22,+der(2),t(2;?)(p23;?),+dup(4)(q12 q21),
+der(11)t(1;11)(q11;q21),+i(12p).

Case 11- 53,X,-Y,-1,-3,-3,-6,+8,+12,-14,+16,-18,-21,-21,-22,
+der(X)t(X;?)(p11;?),+der(1)t(1;?)(p35 or 36;?),
+der del(1)t(1;?)(1q32-->p35 or p36::?),+del(1)(q12),
+del(2)(q34),+der(3)t(3;3)(q21;q27),+del(3)(q21),
+der(3)t(3;7;3?)(3qter-->p21::7q36-->q11::3q25?-->qter?),
+del(5)(p11 or p12),+der dic(7)t(7;17)(q31;p13),
+der(14)t(12;14)(q13;p11),+del(20)(q12),
+der(21)t(21;22)(p11;q11),+M.

Case 12- 104,XY,+X,+X,+Y,+2,+3,+3,+3,+4,+5,+6,+6,+7,+7,+8,+9,+10,+11,
+11,+12,+12,+13,+13,+14,+14,+16,+16,+17,+17,+17,+18,+18,+19,
+19,+20,+20,+20,+21,+21,+21,+21,+22,+inv(1)(p32q21),+i(1p),
+i(1p),+der(2)t(1;2)(q12;q37),+der(2)t(1;2)(q12;q37),
+del(7)(p22),+del(7)(p22),+del(7)(q22),
+der(8)t(8;9)(p23;q12),+der(8)t(8;9)(p23;q12),+i(12p),+i(12p),
+i(12p),+i(12p),+der(15)t(15;?)(p11;?),+M1,+M2.

Case 13- 60,XY,+X,+3,+7,+8,+12,+17,+20,+21,+der(1)t(1;?)(q21;?),+i(12p),
+i(12p),+i(12p),+i(12p),+del(22)(q12).

Case 14- 113,XY,+X,+X,+X,+X,+Y,+1,+2,+2,+2,+3,+3,+4,+5,+5,+6,+6,+6,+6,
+7,+7,+7,+8,+8,+8,+9,+9,+10,+10,+11,+12,+12,+12,+12,+12,+13,
+13,+13,+14,+14,+14,+15,+15,+16,+16,+17,+17,+17,+18,+18,+19,
+19,+20,+20,+21,+21,+21,+21,+22,+22,+der(7)t(7;?)(q11;?),
+der(8)t(8;?)(p21;?),+der(11)t(11;?)(q23;?),+i(12p),+i(12p),
+der(14)t(7;14)(q21;p12),+der(19)t(7;19)(q21;q13),
+dic der(20)t(1;20)(q44;p13).

Table 3. Average number of normal copies of chromosomes and of i(12p) per case

	AVERAGE NUMBER OF NORMAL COPIES OF CHROMOSOMES PER PAIR																								
CASE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Nr. of i(12p)
1	3	2	3	2	3	2	4	4	2	2	2	4	2	2	3	2	3	2	2	3	4	2	2	1	2
2	2	2	2	2	2	3	4	2	2	2	1	3	2	2	2	2	2	2	2	2	2	2	1	1	0
3	3	3	2	2	2	2	3	2	2	2	2	3	2	1	2	2	2	2	2	1	3	2	2	1	2
4	2	3	3	3	2	3	3	3	2	2	1	3	2	3	2	3	3	2	2	2	3	2	3	1	3
5	1	3	3	2	2	3	3	2	2	2	2	3	2	2	2	3	3	2	2	2	4	3	4	1	3
6	2	2	3	2	2	3	2	2	2	2	3	3	2	2	2	3	3	2	2	2	2	2	1	2	3
7	4	4	5	4	3	3	2	5	3	2	2	4	4	4	4	3	3	4	3	6	4	1	4	2	4
8	2	3	2	2	2	3	2	3	2	2	2	3	2	2	3	2	3	2	2	2	2	2	2	2	3
9	2	2	2	2	3	2	3	3	2	2	1	3	2	3	2	2	3	2	2	2	2	2	2	2	3
10	2	1	3	1	2	2	3	4	2	2	1	3	2	3	2	2	3	2	2	4	5	3	2	0	1
11	1	2	0	2	2	1	2	3	2	2	2	3	2	1	2	3	2	1	2	2	0	1	1	0	0
12	2	3	5	3	3	4	4	3	3	3.5	3.5	5	4	4	2	3	4.5	3	4	5	5	3	3	2	4
13	2	2	3	2	2	2	3	3	2	2	2	3	2	2	2	2	3	2	2	3	3	2	2	1	4
14	4	5	4	3	4	5	5	5	4	4	3	6	5	5	4	4	5	4	4	4	6	4	5	2	2

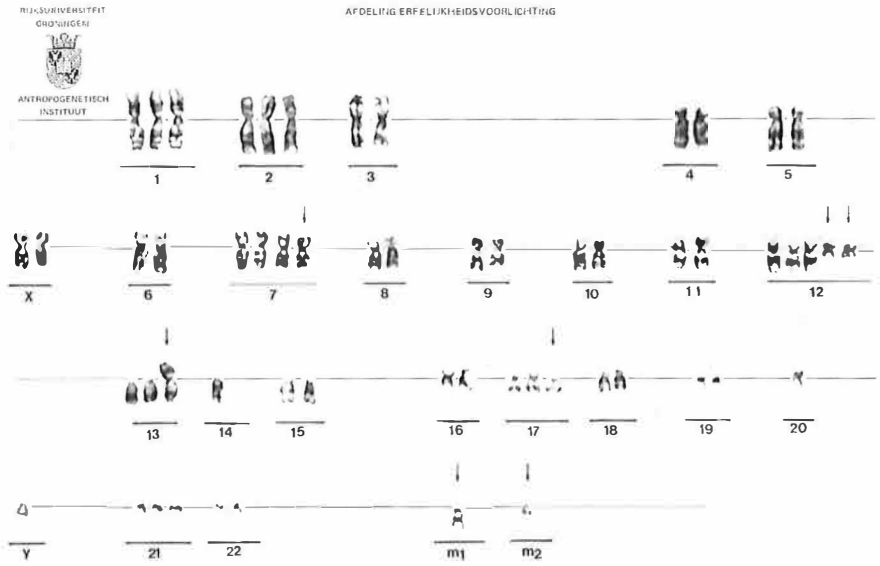

Figure 1. Representative karyotype of Case 3 (the karyotypical description is given in Table 2)

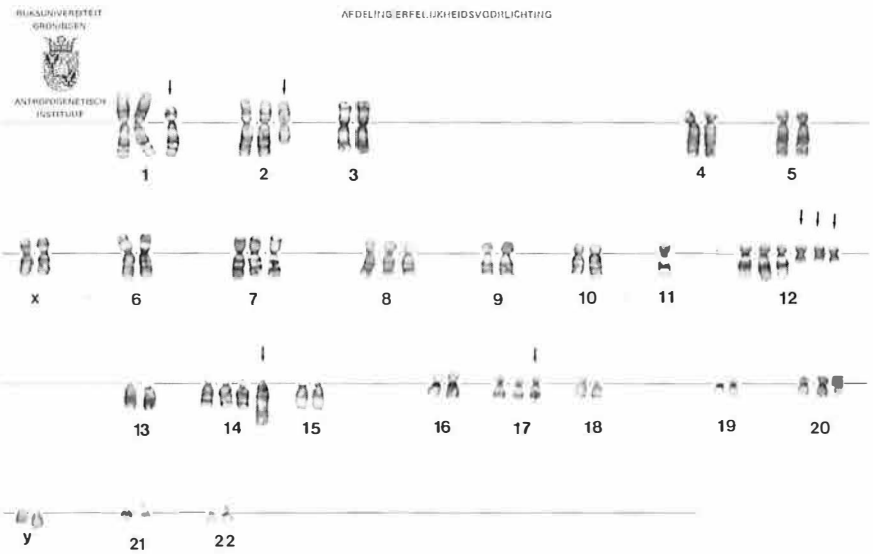

Figure 2. Representative karyotype of Case 9 (the karyotypical description is given in Table 2)

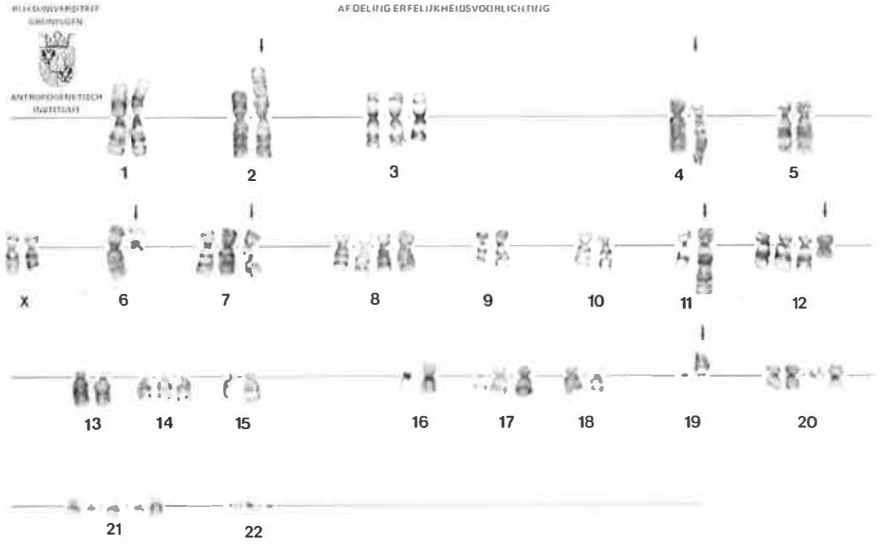

Figure 3. Representative karyotype of Case 10 (the karyotypical description is given in Table 2)

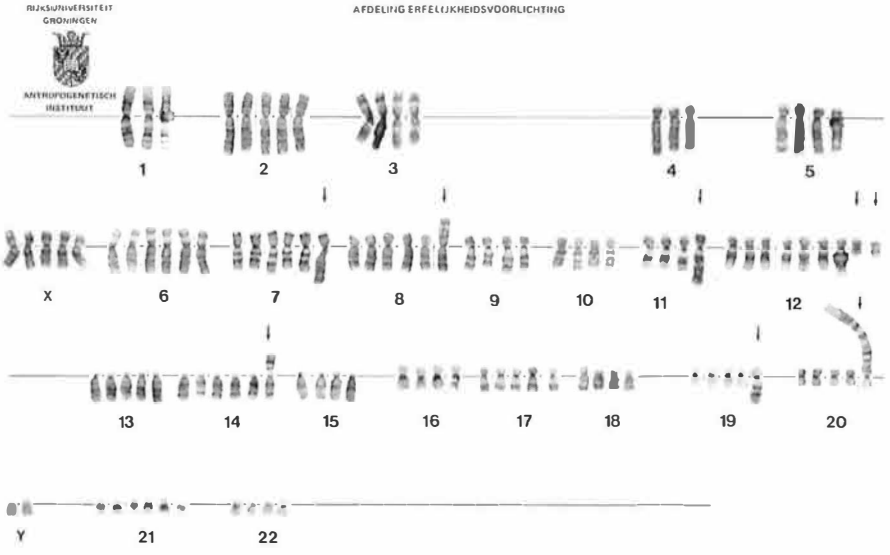

Figure 4. Representative karyotype of Case 14 (the karyotypical description is given in Table 2)

Figure 5 shows the mean chromosome counts combined for all cases, after standardizing the total number of normal chromosomes to the arbitrary number of 46. Multiple comparison using the Newman-Keuls method, shows that the normal copies of chromosomes #12 and X are more frequently found than the normal copies of chromosomes #1, #2, #5, #9, #10, #11, #13, #14, #15, #18, #19, #22, and Y.

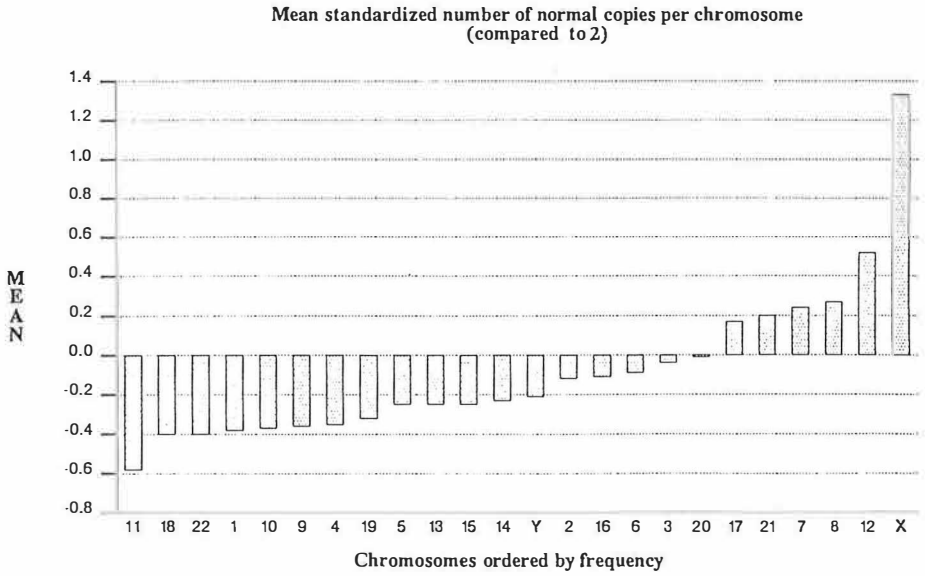

Figure 5 - Average of the standardized number of normal copies of chromosomes per case (compared to 2). Every case was given equal weight in terms of chromosomal counts, which was set arbitrarily to 46.

DISCUSSION

NUMERICAL ABNORMALITIES

Cytogenetic studies of testicular germ cell tumors [16,18-24] have demonstrated the presence of a generally hyperdiploid to hypotriploid chromosome complement, with higher modal chromosome numbers in seminomas than in nonseminomas. Similar results were obtained by measuring the DNA content of the different subtypes of testicular germ cell tumors[1].

Table 4 summarizes the reports found in the literature on chromosomal counts in primary testicular nonseminomas.

---

[1] Oosterhuis JW, Castedo SMMJ, De Jong B, Cornelisse CJ, Dam A, Sleijfer DTh, Koops HS. Ploidy of subtypes of primary germ cell tumors of the testis. Pathogenetic and clinical relevance. (submitted)

Table 4. Summary of the published data on chromosomal counts in primary testicular nonseminomas

AUTHORS	No. OF TUMORS	MODAL CHROMOSOME NUMBERS OR RANGES	AVERAGE OF MODES
Gibas et al. [23]	6	60,81,68,60,58,61	65
Martineau (quoted by [20])	2	78-80,90-114	89
Miles [46]	1	63	63
Rigby [47]	3	52,58,58	56
Galton [22]	5	53-110,61,64,111,111	86
Lelikova [48]	4	53-54,58-65,60,63	59
Atkin [20]	4	57,60,50-58,63	60
Atkin and Baker [37]	1	57-62	60
Saikevitch [24]	1	62	62
PRESENT STUDY	14	65,55,57,65,64,59,102,57,59,60, 53,101,62,113	69

Plotting the number of those tumors against their modal ranges (Figure 6), it is clear that most nonseminomas have between 60 and 64 chromosomes, and only very few are not peritriploid.

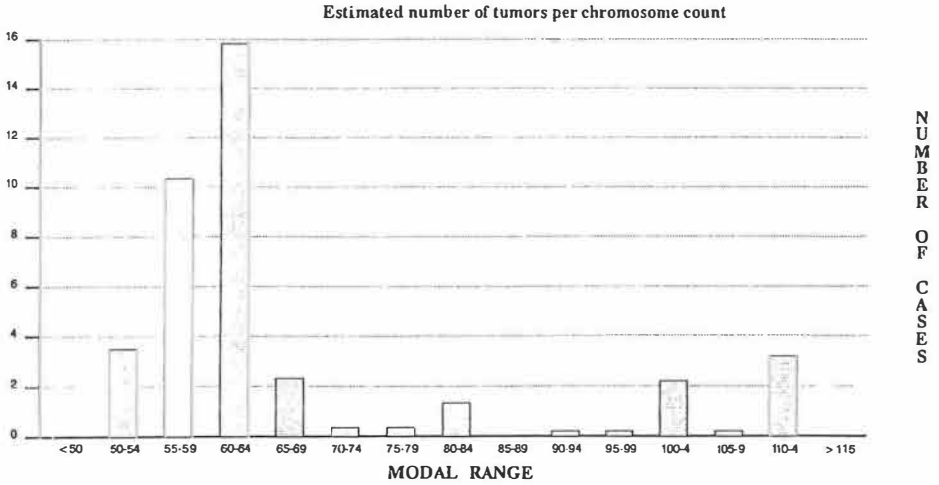

Figure 6. Estimated number of published primary nonseminomas per chromosomal count. When the reported modal counts of a case did not fit in a single class in the graph, the case was plotted as equal fractions in the corresponding classes

Although most seminomas have between 65 to 69 chromosomes, higher numbers are not unusual in these tumors[1].

[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Te Meerman G, Dam A, Koops HS. Cytogenetical analysis of ten seminomas, two of them lacking the i(12p). (submitted)

Several hypotheses have been proposed on the mechanism of origination of aneuploidy in testicular germ cell tumors: successive nondisjunctions of a diploid cell, polyploidization or cell fusion, followed and/or preceded by chromosomal gain and/or loss [25 for review]. If successive nondisjunction of a diploid cell were a mechanism of oncogenesis of nonseminomas, one would expect a higher frequency of near diploid nonseminomas and lower frequencies in higher classes. Since the opposite is observed, there should be other modes of origin of aneuploidy.

The clear peak in the hypotriploid range is in keeping with the measured DNA content of testicular germ cell tumors[1], and can be explained by loss of chromosomes starting from a triploid or tetraploid cell. This would be in keeping with the model of pathogenesis of testicular germ cell tumors proposed by Ewing and Friedman [6,7]. According to this model most testicular germ cell tumors, irrespective of their histology, would originate in a carcinoma in situ cell and progress through a seminoma stage. In disagreement with the tumor progression model proposed by Nowell [26-27], in which the clonal evolution of a tumor cell population goes from diploid to higher chromosome numbers, the progression of testicular germ cell tumors would go from high to lower number of chromosomes, therefore being accompanied by a net loss of chromosomal material. This decrease is probably the end result of the loss of specific chromosomes, the development of structural abnormalities, and the gain of some other chromosomes (or part of chromosomes).

It has been suggested that loss of certain chromosomes or (loss of heterozygosity for) some chromosomal regions is important for the development of malignancy, presumably because of loss of genes with tumor suppressing and differentiation regulating properties [28-36].

If loss of chromosomes in testicular germ cell tumors is related to loss of genes crucial for normal cell differentiation, different chromosomes should be underrepresented in nonseminomas as compared to seminomas. However, since both are germ cell tumors, it should not be surprising that some chromosomes are underrepresented in both subtypes.

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[1] Oosterhuis JW, Castedo SMMJ, De Jong B, Cornelisse CJ, Dam A, Sleijfer DTh, Koops HS. Ploidy of subtypes of primary germ cell tumors of the testis. Pathogenetic and clinical relevance. (submitted)

Tables 2, 3, and Figure 5 give an idea of which chromosomes and part of chromosomes are over- or underrepresented in nonseminomas. As can be seen, #4, #5, #9, #10, #11, #13, #14, #15, #18, #19, #22, and Y are usually underrepresented, whereas #7, #8, #12 (specially its short arm), and X are usually overrepresented. It is of interest that #15 is overrepresented in seminomas[1] and underrepresented in nonseminomas, whilst #17 is underrepresented in the former, but not in the latter. It might be speculated that chromosome 15 contains genes important for sperm cell differentiation. Chromosomes more frequently represented in nonseminomas than in seminomas (e.g. #17) may contain genes responsible for a more malignant development.

The rare occurrence of nonseminomas with chromosome numbers higher than 69 can also be fitted into this model. If loss of heterozygosity for a certain chromosome region plays an important role in the progression of a cell from the seminoma to the nonseminoma stage (which remains to be proven), it will be in the triploid range that chromosome loss will be most critical. As a matter of fact, if a cell contains three copies of a certain autosome, for example two paternal and one maternal, the probability of becoming homozygous for the paternal chromosome through random loss of a single chromosome is 1:3, whereas for a tetraploid cell two random events would be necessary (probability = 1:6). Accordingly, the higher the chromosome number in a seminoma stage cell, the lower the probability of getting a nonseminoma.

STRUCTURAL ABNORMALITIES

As can be seen in Table 2, in primary nonseminomas #12 is very often involved in structural abnormalities, which was also noted by Gibas et al. and DeLozier-Blanchet [21,23]. Twelve out of fourteen tumors had one or more copies of the i(12p), a specific marker for germ cell tumors of the testis [21,23,37,38] and possibly also of the ovary [39,40]. Two tumors, however, lacked that marker. These i(12p) negative testicular germ cell tumors may represent a different group of tumors

[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Te Meerman G, Dam A, Koops HS. Cytogenetical analysis of ten seminomas, two of them lacking the i(12p). (submitted)

with a different clinical evolution[1].

Besides the i(12p) we did not find any structural abnormality in common to different tumors.

As is the case for seminomas [16,[2]], chromosome 1 is also frequently involved in structural abnormalities in nonseminomas (Table 2). In 4 out of 6 primary nonseminomas reported by Gibas [23], and in the case described by Saikevitch [24] there were also structural abnormalities of #1. In cell lines derived from testicular germ cell tumors abnormalities of #1 are also very common [41-43]. Wang et al [42] found a nonrandom involvement of #1 in structural and numerical abnormalities in each of 14 nonseminomatous tumor cell lines. The breakpoints most often found were at 1p12, p22,p36 and q12. The same or contiguous bands were involved in the present series. Chromosome 1 rearrangements, however, have been found in a variety of other solid tumors and in many hematologic malignancies [44 for review]. Yet, as our results show, #1 is much more frequently involved in testicular germ cell tumors than in any other tumor.

Table 6 shows the bands involved in structural abnormalities in the present series, referring to other cytogenetic studies of primary nonseminomas where the same (or contiguous) bands were affected. It is of interest that 22 out of the 73 breakpoints found in our sample have been reported by Gibas [23] and Saikevych [24] in their studies of nonseminomas. Since many breakpoints described (e.g. in #1, #4, #8 and #11) are also found in other kinds of malignancies [44 for review], they should probably be considered proliferation-specific rather than differentiation associated breakpoints [45].

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[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Buist J, Sleijfer DTh. "i(12p) negative germ cell tumors. A different group. (submitted)

[2] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Te Meerman G, Dam A, Koops HS. Cytogenetical analysis of ten seminomas, two of them lacking the i(12p). (submitted)

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CHAPTER V

CYTOGENETICAL STUDY OF A COMBINED GERM CELL TUMOR OF THE TESTIS

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ABSTRACT

The cytogenetical findings in both components of a combined germ cell tumor of the testis are described. The only structural chromosomal abnormality in common was the i(12p).

INTRODUCTION

Combined germ cell tumors of the testis are tumors in which both seminomatous and nonseminomatous components can be identified [1]. Approximately 20% of the germ cell tumors of the adult are combined tumors [1,2].

It is not yet clear whether or not both components have a common origin [9-11]. Cytogenetical analysis of both components of combined germ cell tumors of the testis might prove a powerful tool in the study of the histogenesis of these malignancies.

We report on the cytogenetical analysis of both components of a combined germ cell tumor of the testis.

CASE HISTORY

A 36 year old male presented with a mass, of 3 weeks duration, in the left testis. Ultrasound images showed a tumorous lesion. The serum lactodehydrogenase level was elevated. Alphafetoprotein and beta human choriongonadotrophin levels were in the normal range.

The left testicle was surgically removed. On cross section two separate tumor nodules were found: one (measuring 3x2x2 cm) consisting of whitish, homogeneous tissue, grossly consistent with seminoma; the other (measuring 3x2.5x1.5 cm) consisted of brownish, cystic tissue, grossly consistent with nonseminoma. The nodules were given numbers I and II, respectively. Frozen sections confirmed that lesion I consisted of seminoma and lesion II of embryonal carcinoma. Seminiferous tubules surrounding the tumor contained carcinoma in situ. From both lesions tissue was sampled for cytogenetical studies and for DNA flow cytometry.

MATERIALS AND METHODS

The seminoma component (lesion I) was harvested directly [3]. The nonseminoma component (lesion II) was harvested after short term tissue culture [4].

Measurement of cellular DNA content by flow cytometry [4] was carried out on morphologically checked, representative, fresh samples of

both components. From paraffin-embedded tissue of the tumor additional samples from both components were taken for measurement of cellular DNA content [4]. DNA content is expressed as a DNA index (DI), i.e. the ratio of the tumor and the normal G1 cells (a diploid cell has a DI = 1).

RESULTS

SEMINOMA COMPONENT

Due to a poor metaphase yield, only one metaphase could be fully analyzed. However, 2 additional metaphases had very similar chromosome counts and apparently no structural abnormalities besides i(12p).

The DNA flow graph of this component had one major stemline corresponding to a DI = 2.51 and possibly a minor second stemline corresponding to a DI = 1.86. The chromosomal counts are in keeping with a DI =2.51.

Figure 1 shows the karyotype of the analyzed metaphase, with the following chromosomal constitution:

126,X,-Y,+X,+X,+1,+1,+1,+2,+2,+2,+2,+3,+3,+4,+4,+5,+5,+6,+6,+6,+7,+7,+7, +7,+7,+8,+8,+8,+8,+8,+9,+9,+9,+9,+10,+10,+11,+12,+12,+12,+12,+13,+13, +13,+13,+14,+14,+14,+14,+15,+15,+15,+15,+15,+16,+16,+16,+17,+17,+17, +18,+18,+19,+19,+19,+20,+20,+20,+20,+21,+21,+21,+21,+21,+21,+21,+21, +22,+22,+22,+22,+i(12p),+i(12p).

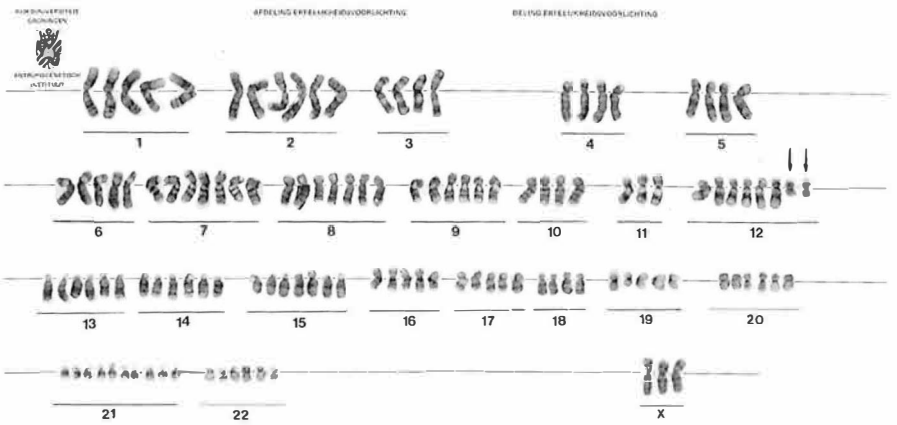


Figure 1. Karyotype of a metaphase from the seminoma component

NONSEMINOMA COMPONENT

The DNA flow graph of the embryonal carcinoma and the intratubular embryonal carcinoma had respectively one rather poorly defined stemline corresponding to a DI around 2, and a small stemline corresponding to a DI = 1.55.

Seven metaphases were analyzed. The chromosomal counts ranged from 72 to 105 (median 101). Figure 2 shows the karyotype of one metaphase of the nonseminoma component, with the following chromosomal constitution:

104,X,Y,+X,+X,+Y,+2,+3,+3,+3,+4,+5,+6,+6,+7,+7,+8,+9,+10,+11,+11,+12,
+12,+13,+13,+14,+14,+16,+16,+17,+17,+17,+18,+18,+19,+19,+20,+20,+20,
+21,+21,+21,+21,+22,+inv(1)(p32 q21),+i(1p),+i(1p),
+der(2)t(1;2)(q12;q37),+der(2)t(1;2)(q12;q37),+del(7)(p22),
+del(7)(p22),+del(7)(q22),+der(8)t(8;9)(p23;q12),
+der(8)t(8;9)(p23;q12),+i(12p),+i(12p),+i(12p),+i(12p),
+der(15)t(15;?)(p11;?),+M1,+M2.

All the structural abnormalities described are clonal.

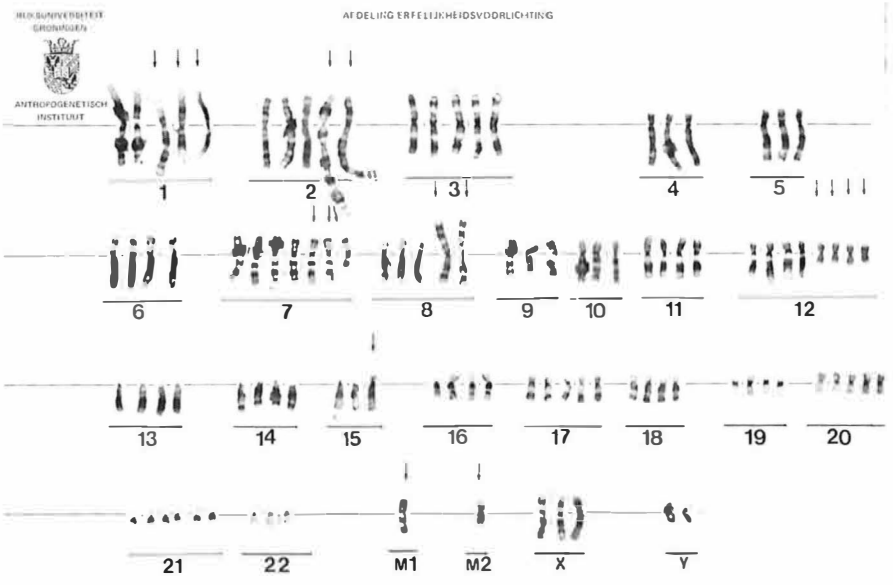


Figure 2. Karyotype of one metaphase from the nonseminoma component

DISCUSSION

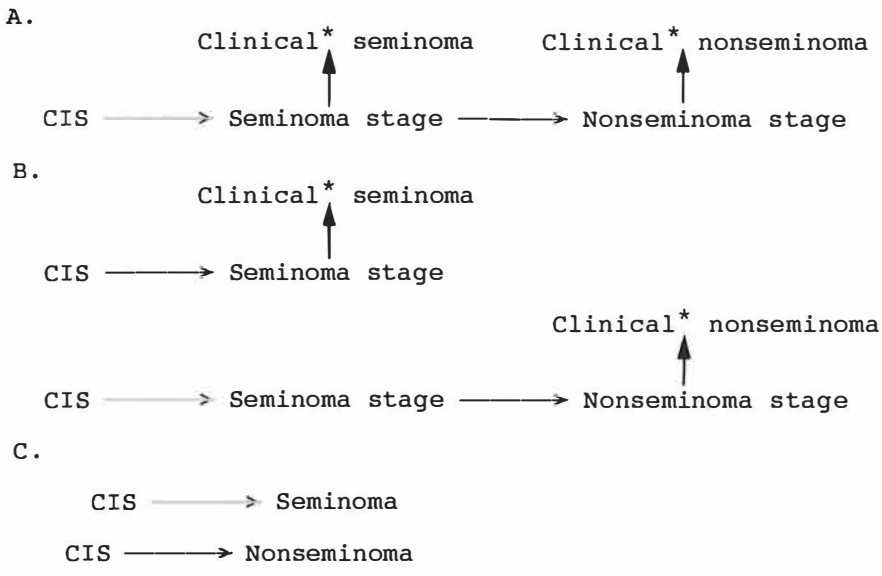
Tumors containing a seminoma and a nonseminoma component are recognized as combined tumors in the British classification [1]. In the

WHO classification they are considered mixed nonseminomas [5]. Both the clinical data and the ploidy of combined germ cell tumors of the testis support the contention that they are a separate entity [1,6].

Little is known regarding the histogenesis of combined germ cell tumors of the testis. It remains controversial whether the seminomatous and nonseminomatous components have a common precursor [9-11].

In the only banded cytogenetical study of a combined germ cell tumors of the testis [7] both components shared one structural chromosomal abnormality besides the i(12p). That finding lends further support on the hypothesis of a common origin of the seminoma and nonseminoma components.

In the present study, however, the only common marker is the i(12p). Since this marker is present in over 80% of all testicular germ cell tumors of the adult [8], it is impossible to derive conclusions regarding the histogenesis of both components. Our findings are compatible with the 3 theoretical possibilities schematically presented in Figure 3. A and B correspond basically to the theories of Ewing [9] and Friedman [10].



Both components would originate from one (in A) or 2 (in B) carcinoma in situ cells, through a seminoma stage. C corresponds to the model of Pierce and Abell [11]. The seminoma and nonseminoma components would

have different origins and independent evolutions.

More cytogenetical studies of combined tumors are needed to derive conclusions about the histogenesis of these malignancies, as well as the relationship between both components. For that purpose, we suggest the use of the technical approach mentioned above, i. e., direct harvesting of the seminomatous component and harvesting after short term tissue culture of the nonseminomatous component. It is our experience that, as compared to seminomas, direct harvesting of nonseminomas will usually yield fewer and poorer metaphases. On the other hand, seminomas do not grow well in vitro [12-14]. Therefore, even in cases where both components are intermingled, direct harvesting tends to select for metaphases of the seminoma component and short term tissue culture will select for nonseminoma cells. DNA flow cytometry will then confirm which component has been karyotyped.

ACKNOWLEDGEMENTS

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We are grateful to Menke Aikema for photographical assistance.

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CHAPTER VI

CHROMOSOMAL CHANGES IN MATURE RESIDUAL TERATOMAS FOLLOWING POLYCHEMOTHERAPY

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ABSTRACT

A cytogenetic analysis of 13 mature residual teratomas following chemotherapy revealed modal chromosome numbers ranging from 52 to 85, in agreement with the flow cytometric determination of the DNA content of the tumors. At least one copy of an i(12p) was present in twelve tumors. One tumor, however, lacked that marker.

The comparison between the chromosomal abnormalities found in mature residual teratomas following chemotherapy and those from primary testicular nonseminomas suggests that residual teratomas result from selection of clones from the primary tumor with a less abnormal karyotype.

INTRODUCTION

Untreated metastases of nonseminomatous germ cell tumors of the testis rarely consist exclusively of fully differentiated, mature somatic tissues [1-4]. However, the residual metastases after polychemotherapy often contain only differentiated teratoma [5-8]. The mechanism(s) involved in this therapy-related differentiation are not yet clear (see [9] for review).

Cytogenetic comparison of mature residual teratomas following chemotherapy with primary testicular nonseminomas may shed light on the mechanism(s) of therapy-related differentiation.

Recently, we described our cytogenetic findings in primary testicular nonseminomas[1]. Here we report on the chromosomal changes in 13 mature teratomas following chemotherapy.

MATERIALS AND METHODS

The tumors were submitted fresh and sterile and were processed for tissue culture and DNA flow cytometry, basically as described [10]. For chromosome preparations the tumor cells were harvested either by brief trypsinization or according to the procedures of Gibas et al. [11]. Colcemid (0.05-0.5 g/ml culture medium) was added two to five hours before harvesting. After harvesting, the cells were centrifuged for 5 minutes at 240 g. The pellets from the trypsinized cells were resuspended in 0.06 M KCL, incubated at 37°C for 15 minutes,

[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Dam A, Te Meerman GJ, Koops HS, Sleijfer DTh. Chromosomal changes in primary testicular nonseminomas. (submitted)

centrifuged, resuspended in a mixture of methanol/acetic acid (3:1), centrifuged, resuspended, and left in the tubes for 20 minutes. The pellets from the cells harvested as described by Gibas et al. were immediately resuspended in fixative. In both methods there was a final centrifugation, after which cells were resuspended and pipetted onto slides and air dried. Chromosomes were GAG and/or GTG banded.

For a statistical evaluation of the chromosomal findings, the number of normal copies per chromosome was analyzed for 13 cases with a two way analysis of variance. The average number of i(12p) per mature residual teratoma was compared with the average number of i(12p) per primary nonseminoma[1] using one sided Mann Whitney U test of significance.

RESULTS

Patient age, number of analyzed metaphases, modal chromosome numbers, and DNA index for each case are given in Table 1.

Table 1. Patient age and cytogenetical data

CASE	PATIENT AGE (yrs)	No. OF ANALYZED METAPHASES	MODAL NUMBER	DNA INDEX
1	32	7	78	1.65
2	27	18	60	1.32/1.21*
3	50	2	62.5	1.45
4	?	13	58	1.26
5	31	3	62	1.37
6	21	4	63	1.39
7	20	14	53	1.15
8	26	6	63	1.39
9	54	9	52	1.22
10	33	9	57	1.23/1.50*
11	24	18	60	1.87
12	30	9	62	NM
13	?	9	57	NM

* Secondary stemline NM not measured

KARYOTYPES

A representative karyotype of each case is described in Table 2.

[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Dam A, Te Meerman GJ, Koops HS, Sleijfer DTh. Chromosomal changes in primary testicular nonseminomas. (submitted)

Table 1. Karyotypical description of a representative metaphase from each case

Case 1	78,X,-Y,+X,+1,+1,+2,+3,+4,+5,+6,+6,+7,+8,+8,+9,+9,+10,+10,+11, +12,+13,+13,+14,+15,+16,+17,+17,+18,+19,+20,+21,+21,+22,+22, +i(12p). (also clonal M(der(18)?)
Case 2	59,XY,+X,+1,+6,+8,+11,+12,+16,+17,+21,+22,+i(12p),+i(12p),+M.
Case 3	62,XY,+X,+1,+3,+5,+6,+7,+9,+10,+12,+16,+17,+19,+21,+i(12p), +i(12p),+del(22)(q12).
Case 4	58,X,-Y,+X,+6,+7,+8,+12,-13,+21,+der(1)t(1;?)(p36;?), +del(2)(q35),+i(12p),+i(12p),+der(17)t(13;17)(q11;q23), +del(22)(q11),+M1,+M2(der(9)?).
Case 5	62,XY,+X,+3,+6,+7,+8,+9,+12,+16,-17,+21,+21,+22, +der(1)t(1;3)(p32;p21),+der(7)t(5;7)(q13;q22),+i(12p),+i(12p), +i(12p),+der(17)t(17;?)(q25;?).
Case 6	65,XY,+X,+1,+2,+3,+6,+7,+7,+8,+8,-10,+12,+13,+17,+20,+21,+22, +del(1)(q41),+der(9)t(9;?)(p13;?),+i(12p),+del(16)(p13),+M1.
Case 7	53,X,-Y,+X,-5,+7,+8,-10,+12,-18,+del(1)(p34), +der(5)t(3;5)(q21;p15),+der(7)t(7;?)(q22;?), +der(10)t(10;?)(q26;?),+del(17)(p11),+M1(18qter-->q11::?),+M2.
Case 8	57,XY,+X,+1,+5,+6,+7,+8,-9,-10,+12,+17,-18,+20,+i(12p), +del(18)(p11),+M1,+M2(9qter-->q11::?),+M3.
Case 9	54,XY,+X,+7,+8,-12,+17,+21,+del(1)(p35?), +der(12)t(12;?)(p13;?), +i(12p),+i(12p).
Case 10	57,XY,+X,-1,+6,+7,-8,+12,-14,+17,+21,+21,+der(1)t(1;?)(p34;?), +der(1)t(1;?)(p11;?),+del(8)(p22),+i(12p),+i(12p),+M1,+M2.
Case 11	87,XY,+X,+1,+1,+2,+2,+3,+3,+3,+4,+5,+8,+8,+9,+9,+10,+10,+12, +12,+13,+13,+14,+14,+15,+16,+17,+17,+18,+19,+20,+20,+21,+21, -22,-22,+der(5)t(5;?)(q31;?),inv(7)(p15 p22), +der(7)t(7;7)(p22;q11),+der(7)t(7;?)(p11;?),del(10)(p13), +der(11)t(11;?)(q25;?),+del(12)(q15 q24),+i(12p),+i(12p), +i(12p),+i(22q),+M1(der(7)?),+M2.
Case 12	64,XY,+X,+Y,+1,+2,+3,+7,+7,+8,+9,+12,+13,+17,+20,+21,+21, +i(12p),+M1(5qter-->q13::?),+M2. (also clonal: del(7)(q31)).
Case 13	57,XY,+2,+3,+5,+7,+8,+12,+16,+17,+der(1)t(1;?)(p36;?),+i(12p), +i(12p).

The numerical abnormalities are given in Table 3. Figures 1-3 show representative karyotypes of, respectively, Cases 4, 10, and 13.

STATISTICAL ANALYSIS

The summary table for the analysis of variance is shown in page 81.

Table 3. Average number of normal copies of chromosomes and of i(12p) per tumor

	AVERAGE NUMBER OF NORMAL COPIES OF CHROMOSOMES PER PAIR																								
CASE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Nr. of i(12p)
1	3.5	3	3	3	3	4	3	4	3.5	4	3	3	4	3	3	3	4	2	3	2.5	4	3	2	0	1
2	2	2	2	2	2	2.5	2.5	2.5	2	2	3	3	2	2	2	3	3	2	2	2	3	3	2	1	2
3	3	3	2.5	2.5	2.5	3	3	2	2.5	3	2.5	2.5	2	2	2.5	2.5	3	2	2.5	2	3	2	2	1	2.5
4	2	2	2	2	2	3	3	3	2	2	2	3	1	2	2	2	2	2	2	2	3	2	2	0	2
5	2	2	3	2	2	3	2	3	3	2	2	3	2	2	2	3	2	2	2	3	4	2	2	1	3
6	3	3	3	2	2	3	4	4	2	1	2	3	2	2	2	2	3	2	2	3	3	3	2	1	1
7	2	2	2	2	1	2	3	3	2	1	2	3	2	2	2	2	2	1	2	2	2	2	2	0	0
8	3	2	2	2	3	3	3	3	1	1	2	3	2	2	2	2	3	1	2	3	2	2	2	1	2
9	2	2	2	2	2	4	3	2.5	2	2	2	1	2	2	1.5	2	3	2	2	2	2	2	2	1	2
10	1	2	2	2	2	3	3	1	2	2	2	3	2	1	2	2	3	2	2	2	4	2	2	1	2
11	4	4	5	3	3	2	1	4	4	2.5	2	4	4	4	3	3	4	3	3	3.5	4	0	2	1	3
12	2	3	3	2	2	2	4.5	2	3	2	2	3	2	2	2.5	3	2	2	2	3	4	2	2	2	1
13	2	3	3	2	3	2	3	3	2	2	2	3	2	2	2	3	3	2	2	2	2	2	1	1	2

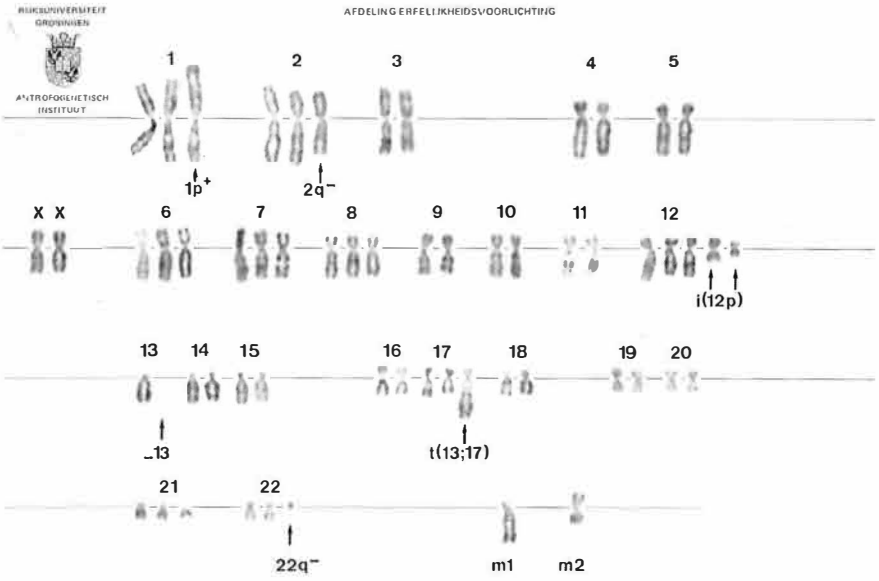

Figure 1. Representative karyotype of Case 4 (the karyotypical description is given in Table 2)

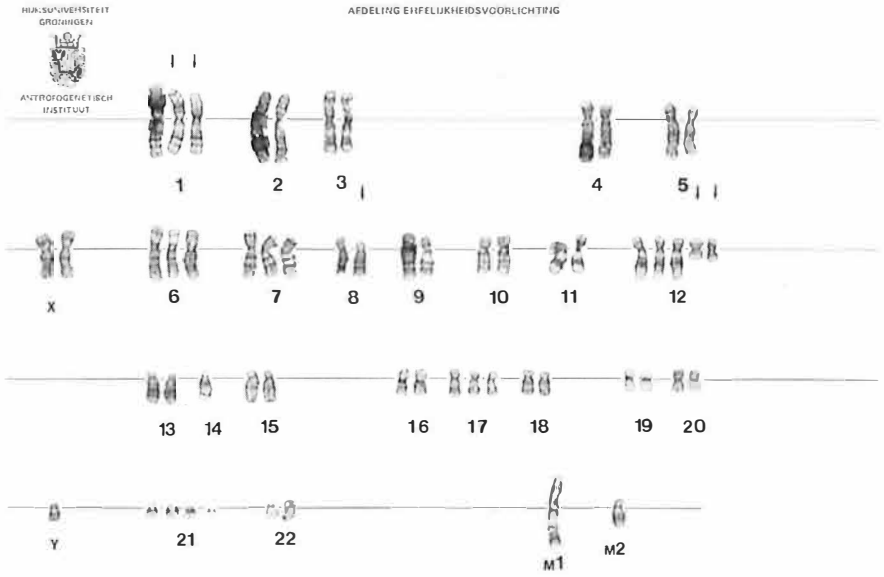

Figure 2. Representative karyotype of Case 10 (the karyotypical description is given in Table 2)

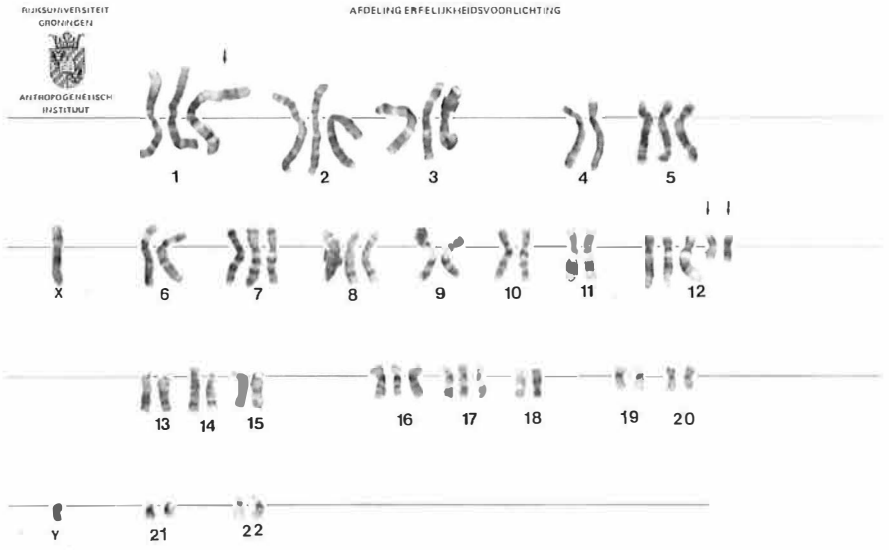

Figure 3. Representative karyotype of Case 13 (the karyotypical description is given in Table 2)

Summary table for the analysis of variance:

EFFECT	SSQUARES	DF	MS	F	P
Chromosomes	62.9	23	2.7	7.1	<0.001
Cases	35.8	12	3.0	7.8	<0.001
Error	105.8	276	0.38		
Total	204.5	311			

All effects are highly significant, indicating that normal copies of chromosomes are present in different numbers and that different individuals have different total numbers of chromosomes. The interaction term, which is used as error term, is numerically rather small, indicating that most of the variability is explained as a combination of differences per person and differences per chromosome.

To indicate the effect of the relative numbers of each chromosome, Figure 4 shows the mean chromosome counts combined for all cases, after standardizing the total number of normal chromosomes to the arbitrary number of 46. Multiple comparison using the Newman-Keuls method, shows that the normal copies of chromosomes #7, #12, #21 and X are more frequently found than the normal copies of chromosomes #10, #14, #18, #22, and Y.

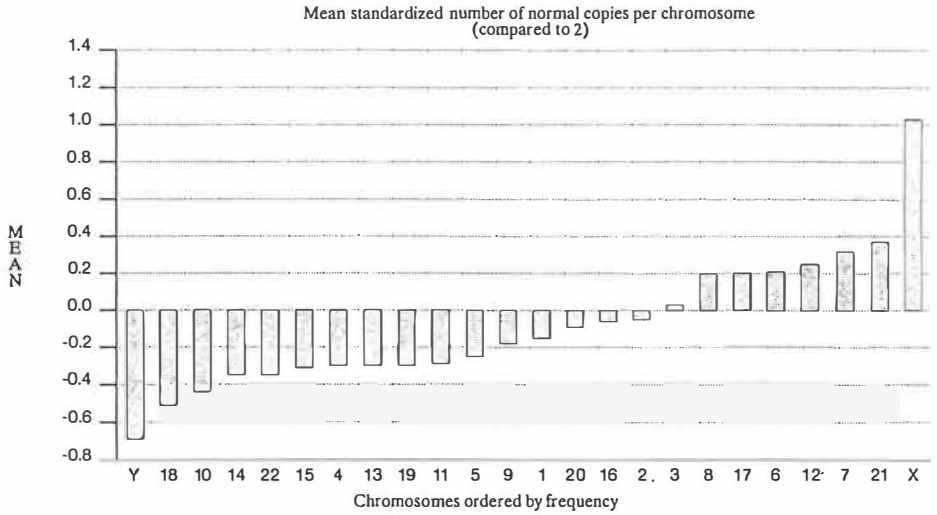

Figure 4 - Average of the standardized number of normal copies of chromosomes per case (compared to 2). Every case was given equal weight in terms of chromosomal counts, which was set arbitrarily to 46.

The one sided Mann Whitney U test showed a significantly ($p<0.005$) lower number of copies of i(12p) in residual teratomas as compared to primary nonseminomas[1].

DISCUSSION

Untreated metastases of nonseminomatous germ cell tumors usually retain the morphologically appearance of the primary tumor (see [12] for review). As is the case in primary nonseminomas, such metastases rarely consist exclusively of fully differentiated mature somatic tissue [1-4]. However, after polychemotherapy there is an apparent shift towards higher degrees of differentiation [5-8]. This can be achieved by three possible mechanisms:

a) Selective destruction of components other than mature teratoma [5,6,8,13].

b) Direct induction of differentiation of malignant cells [6,8,13,14].

c) Spontaneous differentiation of the malignant cells made possible or facilitated by chemotherapy [13].

The mechanisms a) and c) are essentially similar and based on selection:

[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Dam A, Te Meerman GJ, Koops HS, Sleijfer DTh. Chromosomal changes in primary testicular nonseminomas. (submitted)

there is selection of already existing mature teratoma in a) and of cells with an inherent capacity of spontaneously somatic differentiation in c). Thus, actually only two basically different mechanism remain to be considered: induction of differentiation, or selection. These two mechanisms are not mutually exclusive.

Progression of a malignant tumor is the result of clonal evolution of a tumor cell population, and is characterized by an increasing proliferative and malignant potential, and a decreasing capacity of differentiation [15-17].

Another common characteristic of tumor progression is the emergence of new clonal subpopulations that are heterogeneous for a wide variety of genetic, biochemical, enzymatic, immunological, and biological properties [16,21].

In myeloid leukemic cells it has been suggested that, irrespective of their chromosomal constitution, the difference between cells that could and cells that could not be induced to differentiate was controlled by the balance between genes that allow induction of differentiation and genes that suppress differentiation [22,23]. In cells that could not be induced to differentiate chromosomal changes resulting in a different gene balance were able to suppress malignancy by restoring the ability of the cells to (be induced to) differentiate.

Mature residual teratoma following intensive chemotherapy of nonseminomas might be the result of selection of tumor cells with an abnormal but balanced chromosomal constitution that still would allow spontaneous or induced differentiation. The selected cells might either belong to early evolutionary clones (with a low malignant potential and inherent capacity of somatic differentiation) that have been overgrown by more malignant ones, or from later, more malignant clones which obtained through loss and or gain of specific chromosomes the right balance to make possible (induction of) differentiation. Alternatively, the mature residual tumor tissue may be the result of induction of differentiation in tumor cells irrespective of their chromosomal pattern and inherent capacity to differentiate.

If mature residual teratomas are the result of differentiation of selected cells with a specific balanced chromosomal pattern, one might expect specific differences between the chromosomal constitutions of primary nonseminomas and residual teratomas. However, if induction of differentiation is possible irrespective of the chromosomal pattern, no

such differences would be expected.

As was the case in primary nonseminomas, most residual teratomas have between 60 and 64 chromosomes, in agreement with the flow cytometric determination of the DNA content of these tumors (Table 1). However, residual teratomas differ from primary nonseminomas in the chromosomes under- and overrepresented. In residual teratomas the underrepresentation of chromosome Y is greater than in primary nonseminomas[1], whereas the underrepresentation of #9 and #11, as well as the overrepresentation of #12 and X are smaller than in primary nonseminomas. Similar discrepancies were observed in two different cases where both the primary tumors and the residual teratomas were karyotyped [24,[2]]. Although these differences are difficult to interpret in such a small sample, it is conceivable that chromosomes present in residual teratomas in higher numbers than in primary nonseminomas contain genes important for normal differentiation, whereas chromosomes present in higher numbers in primary nonseminomas may contain genes responsible for a more malignant development.

As can be seen in Table 2, #12 is the chromosome most often involved in structural abnormalities in residual teratomas, as previously noted in seminomas [25,[3]] and primary nonseminomas [26,27,[1]]. Twelve out of thirteen tumors had one or more copies of the i(12p), a specific marker for germ cell tumors of the testis [25-29] and possibly also of the ovary [30,31]. It is of interest that the average number of i(12p) per tumor is significantly smaller in residual teratomas (1.6) than in primary nonseminomas (2.3). This finding is in agreement with the contention that the number of copies of i(12p) correlates with an increased malignancy [27].

At variance with the cytogenetic findings in primary nonseminomas [17,18,[1]] and seminomas [16,[3]], only 7 out of 13 residual teratomas had rearrangements of #1 (as opposed to 13 out 14 in primary nonseminomas),

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[1] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Idenburg VJS, Dam A, Te Meerman GJ, Koops HS, Sleijfer DTh. Chromosomal changes in primary testicular nonseminomas. (submitted)

[2] De Jong B, et al. Unpublished data.

[3] Castedo SMMJ, De Jong B, Oosterhuis JW, Seruca R, Te Meerman GJ, Dam A, Koops HS. Cytogenetical analysis of 10 seminomas, two of them lacking the i(12p). (submitted)

and only one with a breakpoint at 1q. Moreover, in the present series of residual teratomas we noted 61 different markers and 36 different breakpoints (Table 2), as opposed to, respectively, 91, and 73 in primary nonseminomas[1]. It is also remarkable that structural abnormalities, usually considered signs of expression of malignancy, are less frequent in residual teratomas (average=4.7) than in primary nonseminomas (average:6.5[1]). On the other hand, 25 out of the 36 different bands involved in structural rearrangements in residual teratomas were also implicated in primary nonseminomas[1], which stresses the relationship between both groups.

Thus, our findings suggest that residual teratomas following intensive chemotherapy are the result of selection of clones with a less abnormal karyotype and possibly the right balance of genes allowing differentiation.

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CHAPTER VII

A RESIDUAL MATURE TERATOMA WITH A MORE BALANCED KARYOTYPE THAN THE PRIMARY TESTICULAR NONSEMINOMA?

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SUMMARY

We have been able to study the karyotypes and measure the DNA content in both the primary nonseminomatous germ cell tumor of the testis and the residual mature teratoma after chemotherapy of the same patient.

Based on these and other studies the mechanism of therapy related differentiation in germ cell tumors of the testes is discussed.

INTRODUCTION

Comparison of the karyotypes of primary testicular nonseminomas and the karyotypes of the residual mature teratoma (RMT) following chemotherapy of its metastases, might shed light on the mechanism of therapy related differentiation. Here we report the first patient, in whom both the primary testicular nonseminoma and the residual mature teratoma were karyotyped.

CASE HISTORY

A 23 year old man had a tumor measuring 8x5x4 cm in his left testicle, which was removed surgically. The tumor was a nonseminomatous germ cell tumor with the following intermingled components: embryonal carcinoma, immature and mature teratoma, small foci of yolk sac tumor and scattered trophoblastic giant cells. Alpha fetoprotein (AFP) and beta human choriogonadotropin (HCG) were demonstrated immunohistochemically respectively in the last two components.

The patient had one retroperitoneal and two lung metastases; serum AFP and HCG were elevated. Following chemotherapy according to the European Organization for Research and Treatment of Cancer (EORTC) protocol 30824 (alternatingly bleomycin + etoposide + cisplatin (BEP) and cisplatin + vinblastine + bleomycin (PVB)), the marker levels normalized according to half life and the lung metastasis disappeared. A retroperitoneal residual mass (8x5x2 cm) was surgically removed. It consisted of mature teratoma only.

MATERIALS AND METHODS

The following procedures were carried out on morphologically checked, representative, fresh samples of the primary tumor and the RMT: short term tissue culture, karyotyping and measurement of cellular DNA content using flow cytometry, as previously described [1]. From the

paraffin embedded tissue of the primary tumor three additional samples were taken for measurement of cellular DNA content.

Cellular DNA content is expressed as DNA index (DI), i.e. the ratio of the tumor and the normal G1 (a diploid cell has DI = 1).

RESULTS

CELLULAR DNA CONTENT

The different components in the primary tumor were too tightly interwoven to allow separate measurement of their DI. The mixed tumor, however, showed one rather broad aneuploid peak (fig. 1-A), with DI = 2.23. A single aneuploid stemline was found in the paraffin-embedded tumor tissue, also with DI = 2.27, not significantly different from the DI in the fresh sample. The RMT also showed only one aneuploid peak DI = 1.87 (fig. 1-B).

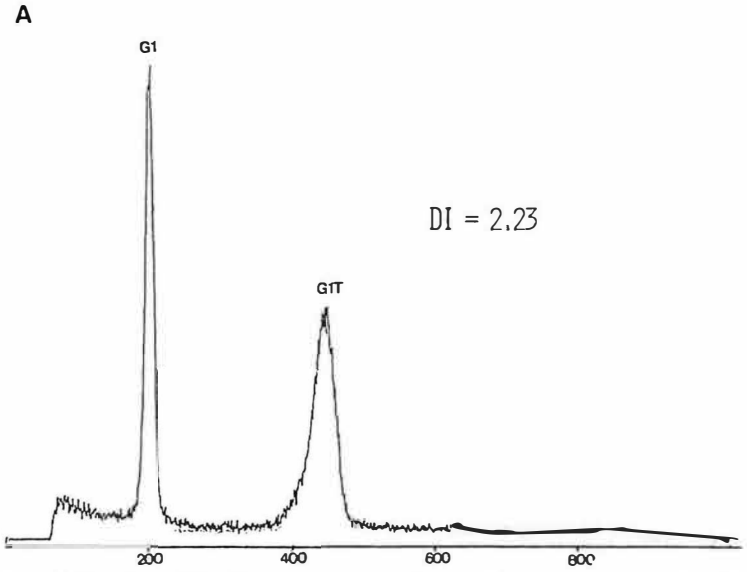

Figure 1 - (A) The DNA flow graph of the primary tumor with DI = 2.23 in agreement with its modal chromosome number (102).

These data are in agreement with the modal chromosome numbers found in the primary tumor and the RMT: about 102 and 86, respectively. Apparently the karyotypes are representative for main aneuploid stemlines.

KARYOTYPING

We analyzed (or partly analyzed) 24 metaphases from the primary tumor and 17 from the RMT.

In the primary tumor we found three different related clones with chromosome numbers between 94 and 107 (mean 102).

Fig. 2 shows one of the karyotypes with the following chromosomal pattern (description made comparing to a tetraploid cell):

102,XXYY,+X,+X,+3,-5,-7,+8,-10,-11,-11,-11,-16,-17,-19,+20,+20,-22,
-22,-22,+der(1)t(1;?)(p13;?),+del(3)(p23),+der(5)t(5;?)(q31:?),
del(6)(q21),inv(7)(p15 p22),+der(7)t(7;7)(p15;q11),
+del(8)(p12),del(9)(p11),del(10)(p13),+der(11)t(11;14)(q14;q11),
der(11)t(11;?)(q25;?),+del(12)(q15 q24),+del(12)(q15 q24),
+i(12p),+i(12p),+i(12p),+i(12p),+i(22q),+i(22q),+m1 (der(7)?).

In the RMT we found closely related chromosomal patterns, probably belonging to one clone, with chromosome numbers ranging from 83-88 (mean 86).

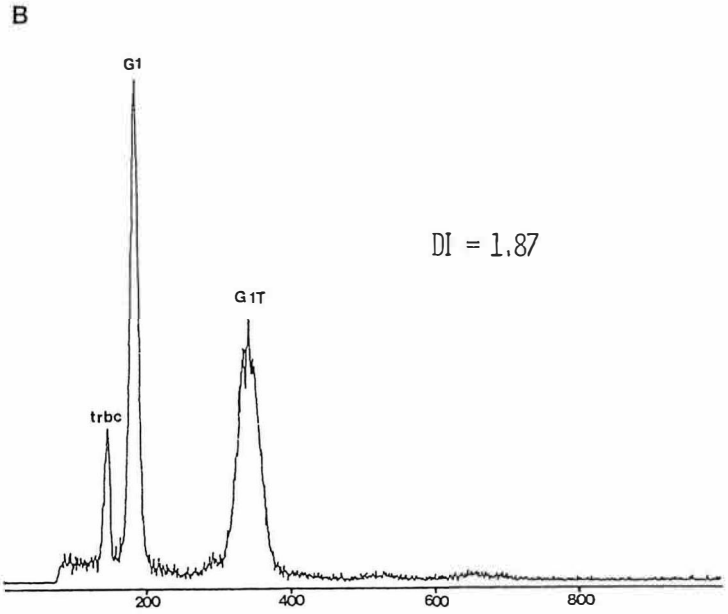

Figure 1 (cont.) - (B) shows the DNA flow graph of the residual mature teratoma with DI = 1.87 in agreement with its modal chromosome number (86).

Fig. 3 shows one of the karyotypes with the following chromosomal constitution (the above referred method of description is followed):

87,XXY,-Y,+3,-4,-5,-6,-6,-7,-7,-11,-11,-15,-16,-18,-19,-22,-22,
-22,-22,+der(5)t(5;?)(q31;?)*,inv(7)(p15 p22)*,
+der(7)t(7;7)(p22;q11),+der(7)t(7;?)(p11;?),
del(10)(p13)*,+der(11)t(11;?)(q25;?)*,+del(12)(q15 q24)*,
+i(12p)*,+i(12p)*,+i(12p)*,+i(22q)*,+m1 (der(7)?),+m2.

The chromosomal abnormalities that are shared by the primary tumor

and the RMT are indicated by an asterisk.

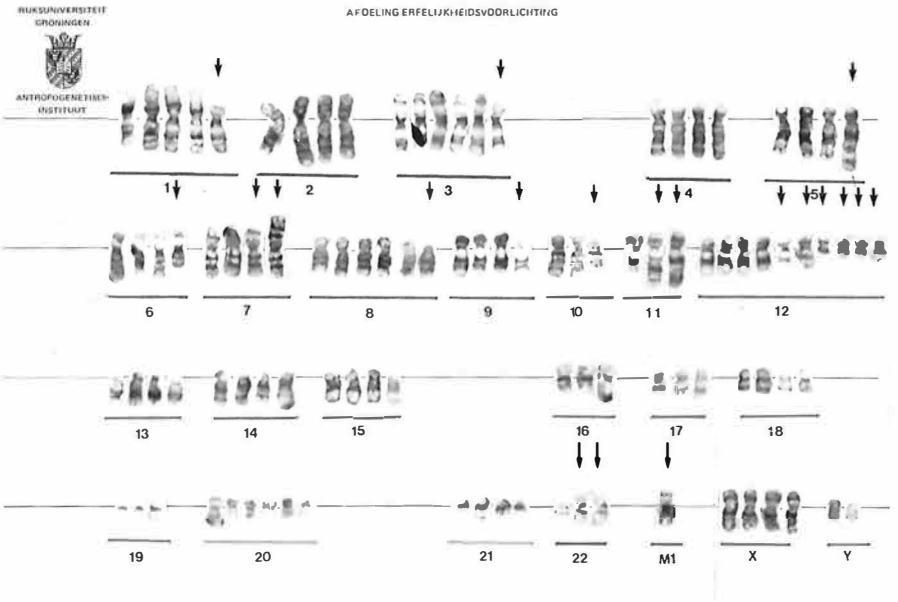

Figure 2 - Karyotype of one metaphase of the primary tumor with arrows pointing to the chromosomal abnormalities

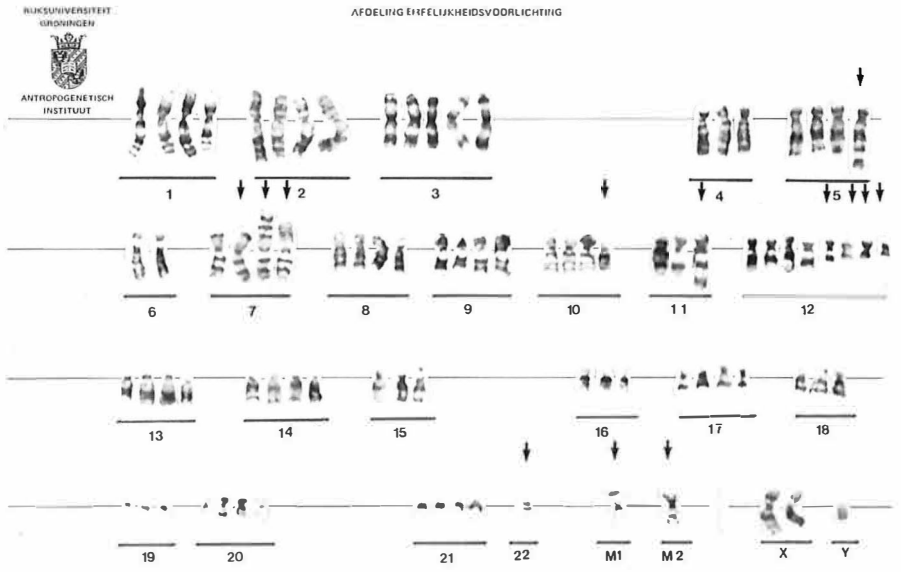

Figure 3 - Karyotype of one metaphase of the RMT with arrows pointing to the chromosomal abnormalities

DISCUSSION

Comparison of the karyotypes of the primary tumor and its metastases shows that they are related and share several chromosomal abnormalities. On the other hand, the DNA flow graph of the sample of the primary tumor, with an admittedly small, mature teratoma component in it, does not show a stemline corresponding with the stemline of the RMT. Two explanations are possible:

1. The clone in the primary tumor from which the RMT originated was missed due to inadequate sampling, or the clone was too small to be detected. Additional thorough sampling of the primary tumor from the paraffin-embedded tissue did not reveal another stemline with the same DI as the RMT.

2. Alternatively, the clone was not present in the primary tumor but developed only in the metastasis.

The available data do not allow a decision between these two possibilities. The clone of the RMT may have been present in the primary tumor as an evolutionary early clone, which was subsequently overgrown by later clones with higher chromosome numbers. It is also possible that the clone of the RMT developed at a later stage from the clone in the primary tumor through loss of chromosomes.

The clone of the RMT apparently has a chromosomal constitution with a proper balance between genes allowing and suppressing differentiation [2]. We have shown that residual mature teratoma following chemotherapy of disseminated nonseminomatous germ cell tumors of the testis is associated with primary tumors containing a mature teratoma component [3]. Unless a metastatic tumor has an inherent capacity for spontaneous somatic differentiation, which it has in common with the primary tumor, chemotherapy will not produce residual mature teratoma, but rather necrosis and fibrosis. This finding led us to conclude that selection of differentiating clones plays the major role in chemotherapy-related differentiation of metastatic germ cell tumors, and not chemotherapeutic de novo induction of differentiation. Using cisplatinum in vitro and in mouse teratocarcinoma models, we collected experimental evidence to support this conclusion [ref. 4, for review]. With the newer chemotherapeutic regimen BEP, induction of differentiation is reconsidered [5].

We have also shown that mature residual teratomas, for all their high degree of somatic differentiation, are composed of highly aneuploid

tumor cells, with karyotypes containing, among many other abnormalities, the metacentric marker chromosome characteristic for germ cell tumors of testis and ovary [1,6-11].

The present results are in agreement with our previous findings. They lend further support to the hypothesis that chemotherapy selects for tumor cells that are either already differentiated or are particularly prone to somatic differentiation.

Sachs and coworkers, studying the chromosomes of myeloid leukemic cells, showed that the change from the inability to the ability of cells to be induced to differentiate to mature nondividing cells by a normal differentiation inducer, is controlled by the balance between genes that allow induction of differentiation and genes that suppress differentiation [2]. Chromosome alterations resulting in changes in gene balance are able to suppress malignancy by restoring the ability of the cells to be induced to differentiate thereby bypassing preexisting genetic changes. It is even possible to obtain normal growth control without induction of differentiation by restoring the normal balance between genes for expression and suppression of malignancy [2]. The finding of karyotypes in tumor cells from RMT that are related but not identical to the karyotype of the primary tumor supports the idea that RMT is composed of cells selected for the right balance of genes, allowing either spontaneous or therapy-related differentiation.

Careful analysis of more cases in which both primary tumor and RMT have been karyotyped will also resolve the question whether we are dealing with an already existing clone in the primary tumor or with a newly developed clone in the metastatic lesion. Such a comparison of the karyotypes will probably also tell which chromosomes or combinations of chromosomes are important for somatic differentiation in testicular germ cell tumors.

ACKNOWLEDGEMENTS

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CHAPTER VIII

"i(12p) NEGATIVE" TESTICULAR GERM CELL TUMORS. A DIFFERENT GROUP?

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ABSTRACT

We report on the cytogenetical analysis of three seminomas, two primary nonseminomas and two mature residual teratomas following chemotherapy all lacking the i(12p).

Testicular germ cell tumors without an i(12p) may represent a different group of germ cell tumors, also in their clinical course, as compared to those having the i(12p).

INTRODUCTION

In 1982 Atkin and Baker [1] reported the existence of an i(12p) as a specific chromosome abnormality in testicular germ cell tumors (TGCT). Further studies [2-8] confirmed their observations.

Besides our recent description of two seminomas lacking the i(12p) [9], we are not aware of other reports that firmly deny the presence of an i(12p) on TGCT.

In a study of well banded chromosome preparations of 42 TGCT, we found that 3 out of 13 seminomas, 2 out of 15 primary nonseminomas, and 2 out of 14 residual mature teratomas clearly lacked the i(12p).
Since the i(12p) supposedly plays an important role in the oncogenesis of TGCT, "i(12p) negative" TGCT may represent a different group of germ cell tumors.

MATERIAL AND METHODS

Direct harvesting of tumor cells for karyotyping [3] was carried out on all seminomas. Both primary nonseminomas and mature residual teratomas have been harvested after short term tissue culture [6]. Case 5 (combined tumor) was divided in 2 parts, one for direct harvesting and the other for short term tissue culture.

The measurement of cellular DNA content by flow cytometry was carried out on morphologically checked, representative, fresh samples, as well as on paraffin-embedded tissue of the tumors [6]. DNA content is expressed as a DNA index (DI), i.e. the ratio between tumor cells and normal G1 cells (a diploid cell has a DI = 1).

Histological classification of all tumors was done prior to the cytogenetical study. Slides were checked for the correct classification after knowing the karyotypes. First a group of i(12p) positive and negative TGCT was analyzed not knowing to which group the tumor belonged, and then with the full cytogenetical information.

RESULTS

Data concerning patient age, clinical stage, tumor histology and modal chromosome number are summarized in Table 1.

Table 1. Summary of the clinical, histopathological and cytogenetical data

i(12p) NEGATIVE GERM CELL TUMORS

CASE	PATIENT AGE (yrs)	CLINICAL STAGE #	HISTOLOGY	DNA INDEX	CHROMOSOME NUMBER
1	31	I	CLASSICAL SEMINOMA	2.5	106
2	43	I	CLASSICAL SEMINOMA	1.58	71
3	44	I	CLASSICAL SEMINOMA	2.6	109
4	24	I	NONSEMINOMA (EC/IT/MT)	1.27	55
5	64	II-C	COMBINED TUMOR (SE/EC/IT/MT)	1.68*/1.33**	53
6	40	I§	MATURE TERATOMA	1.15	53
7	29	II§§	MATURE TERATOMA	1.35	56

At presentation SE- seminoma; EC- embryonal cell carcinoma; IT- immature teratoma; MT- mature teratoma * DI of the seminoma component ** DI of the nonseminoma component § During follow up II-B §§ During follow up IV

KARYOTYPING

In the following karyotypical descriptions, the number of metaphases analyzed refers only to abnormal metaphases. From all cases a variable number of normal metaphases was also analyzed. All metaphases analyzed clearly lacked the i(12p).

CASE 1 (Primary seminoma)

This case was described previously [9]. Ten metaphases were analyzed. The modal chromosome number was 106. In all metaphases analyzed the same markers were present.

Figure 1 shows the karyotype of one of the cells with the following chromosomal constitution:

106,X,-Y,+X,+X,+1,+1,+2,+2,+2,+3,+4,+4,+5,+6,+6,+6,+7,+7,+7,
+7,+8,+8,+8,+8,+9,+9,+9,+10,+11,+12,+12,+12,+13,+14,+14,
+15,+15,+15,+16,+16,+17,+18,+18,+19,+19,+20,+20,+20,+21,
+21,+21,+21,+21,+22,+22,+22,+22,+der(1)t(1;?)(p11;?),
+i(2q),+der(3)t(3;?)(p23;?),+del(12)(q24.2),
+der(15)t(15;?)(q22;?),+der(15)t(15;?)(q22;?).

CASE 2 (Primary seminoma)

Case 2 was also described previously [9]. Seven metaphases were analyzed. The modal chromosome number was 71.

A karyotype representative for all markers found in this case is:

63,XY,+3,-4,+7,+8,+8,+9,+10,+12,+15,+15,+16,+17,+21,+21,
+21,+22,+22,+der(4)t(1;4)(q21;q34),+der(12)t(12;?)(p12;?)

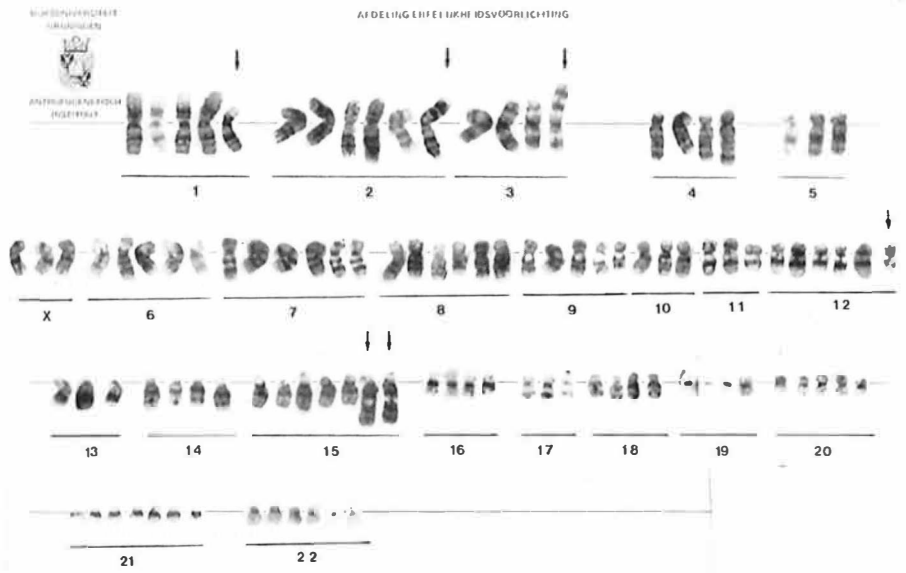

Figure 1 - Karyotype of one metaphase of Case 1 (primary seminoma).

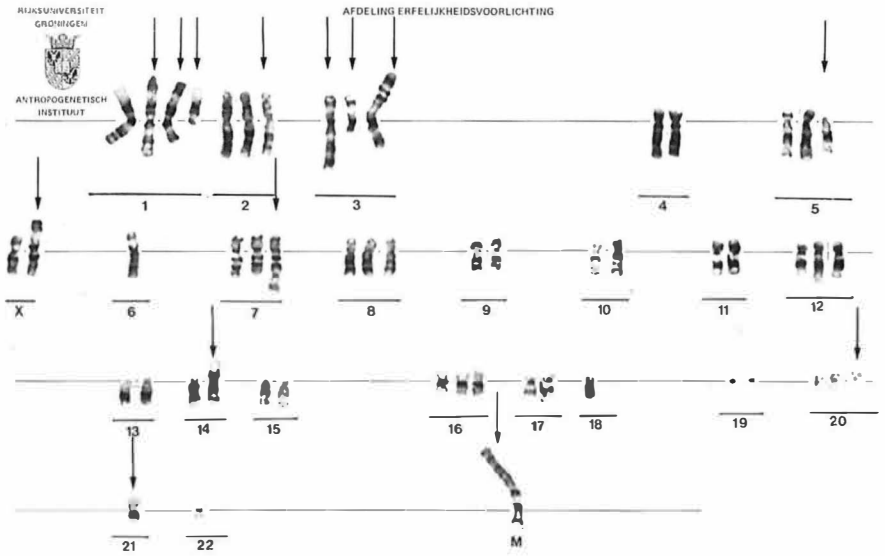

Figure 2 - Karyotype of one metaphase of Case 5 (combined tumor)

CASE 3 (Primary seminoma)

Ten metaphases were analyzed, with chromosomal counts ranging from 100 to 120 (median 109). A representative karyotype of this case was:

113,XY,+X,+X,+1,+1,+2,+2,+2,+2,+2,+3,+3,+3,+3,+4,+4,+5,+5,+6,+6,
+7,+8,+8,+8,+8,+8,+9,+10,+10,+12,+12,+12,+13,+14,+14,+14,
+15,+15,+16,+16,+19,+19,+20,+20,+20,+20,+20,+21,+21,+21,
+22,+22,+22,+22,+22,+i(1q),+der(7)t(7;?)(q22;?),+i(7p),
+i(7p),+i(7p),+der(12)t(12;?)(q13;?),+der(12)t(12;?)(p11;?),
+i(17q),+M1,+M2(der(12)?),+M3(der(1p)),+M4,+M5.

CASE 4 (Primary nonseminoma)

Nine metaphases were analyzed, with a modal chromosome number of 55. The modal karyotype was:

55,XY,+6,+7,+7,-11,+12,+der(2)t(1;2)(p32;q35),+der(3)t(3;4?)(p11;p11?),
+der(8)t(8;?)(q24;?),+der(11)t(11;?)(q25;?),
+der(17)t(17;?)(p11;?),+der(21)t(1;21)(q12;p11).

CASE 5 (Combined tumor)

From this case eight metaphases were analyzed (three from the directly harvested tumor and five from the cultured material). The karyotypes of all metaphases were very similar, with chromosomal counts ranging from 50 to 55 (mode 53). Although the seminoma and nonseminoma components were too intermingled to allow a separate harvesting, it was possible to measure separately the DNA content of both.

We are convinced that we have karyotyped the nonseminoma component, because tumor cells were easily kept in culture, which does not happen with seminoma cells. Besides, the DI of the nonseminoma component was in keeping with the chromosomal findings.

Figure 2 shows a representative karyotype of a metaphase from this case, with the following karyotype:

53,X,-Y,-1,-3,-3,-6,+8,+12,-14,+16,-18,-21,-21,-22,+der(X)t(X;?)(p11;?),
+der(1)t(1;?)(p35 or 36;?),+der del(1)t(1;?)(1q32-->p35 or p36::?),
+del(1)(q12),+del(2)(q34),+der(3)t(3;3)(q21;q27),+del(3)(q21),
+der(3)t(3;7;3?)(3qter-->p21::7q36-->q11::3q25?-->qter?),
+del(5)(p11 or p12),+der dic(7)t(7;17)(q31;p13),
+der(14)t(12;14)(q13;p11),+del(20)(q12),
+der(21)t(21;22)(p11;q11),+M.

The unidentifiable segment translocated to the short arm of both der(1) is probably identical to the terminal part of the short arm of the der(X).

CASE 6 (Mature residual teratoma)

Forteen metaphases were analyzed, all sharing the same markers. The modal chromosome number was 53. Figure 3 shows a karyotype from this

case, with the following description:

53,X,-Y,+X,-5,+7,+8,-10,+12,-18,+del(1)(p34),+der(5)t(3;5)(q21;p15),
+der(7)t(7;?)(q22;?),+der(10)t(10;?)(q26;?),+del(17)(p11),
+M1(der(18q)),+M2.

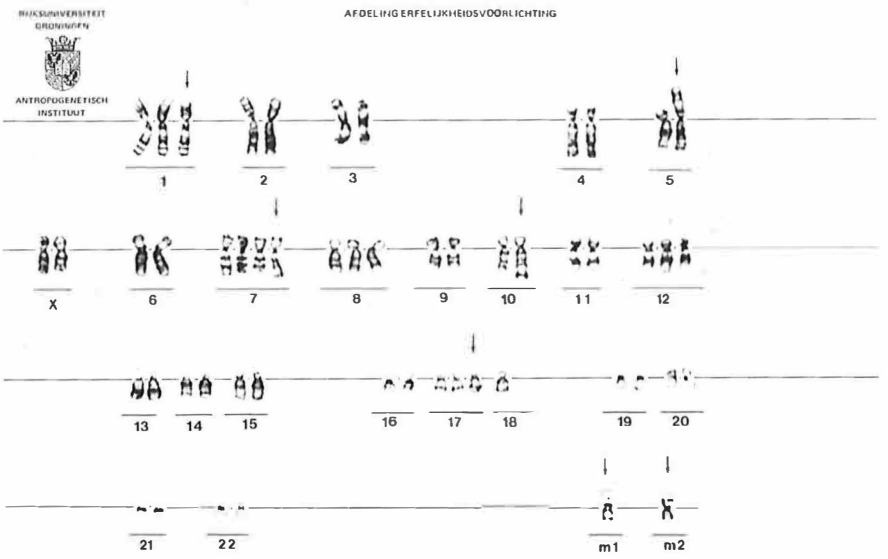

Figure 3 - Karyotype of one metaphase of Case 6 (mature residual teratoma)

CASE 7 (Mature residual teratoma)

Only 5 metaphases could be analyzed. The chromosomal counts ranged from 50 to 59 (median 56).

A karyotype representative for all structural abnormalities present in this case is:

59,XY,+X?,+1,-2,-2,+7,+8,-9,-9,+10,-11,+12,+20,+der(2)t(2;?)(q11;?),
+der(2)t(2;?)(p11;?),+der(3)t(2;3)(q11;p13),+del(6)(q21),
+del(7)(q22),+der(11)t(11;?)(q22;?),+der(14)t(14;?)(p11;?),
+2M1,+M2,+M3.

DISCUSSION

After the first reports of Atkin and Baker on the existence of an i(12p) in TGCT [1,2], further studies [3-8] confirmed that i(12p) was a specific marker for all types of TGCT. Recently the same marker was also found in ovarian cancer [10-11]. Therefore it seems possible that the i(12p) is important in the development of gonadal germ cell tumors in general. However, in the few cytogenetical studies of extragonadal

germ cell tumors no i(12p) has been found [12,13]. We have recently karyotyped another mediastinal germ cell tumor also lacking that marker (paper in preparation).

The present study clearly demonstrates that there are also TGCT without an i(12p). To compare the average number of normal copies of chromosome 12 found in i(12p) positive and negative TGCT we have given each tumor the same weight, irrespective of its total chromosome count, by setting it arbitrarily to 46. The average number of normal copies of #12 in the i(12p) positive TGCT was about 2.13, in the i(12p) negative TGCT about 2.27. Thus, the number of normal copies of chromosome 12 is approximately the same in both groups of tumors. Besides the i(12p) an excess of normal copies of chromosome 12 has been frequently found in TGCT [1,3,9]. This supports the speculation that overrepresentation of genes localized in 12p may play a critical role in the oncogenesis and/or tumor progression of TGCT [1-7,9]. The discussion on other cytogenetical findings in TGCT is given elsewhere (9, and paper in preparation).

So far, the clinical evolution of patients 1 to 6 has been uncharacteristic. However, concerning case 7 it is of interest that 11 months following removal of the retroperitoneal residual mature teratoma, a secondary non-germ cell tumor was resected from the retroperitoneum. This malignancy was histologically classified as a small cell carcinoma with neuroendocrine features. The primary tumor and the residual mature teratoma after chemotherapy lacked such a small cell carcinoma component. The non-germ cell malignancy apparently originated in the residual teratoma, as previously described [14].

In the past 10 years we have diagnosed over 40 residual teratomas. A secondary non-germ cell malignancy in a residual mature teratoma is a rare event, since we found it in only three cases. Absence of i(12p) is also a rare event in residual mature teratoma (two cases out of 14). Yet one of the two residual mature teratomas lacking i(12p) developed a secondary malignancy. Although this finding may be coincidental, one should consider the possibility that i(12p) positive and negative TGCT represent different types of tumors, with a different clinical behaviour.

Compared to TGCT mediastinal germ cell tumors carry a higher risk of developing a secondary non-germ cell malignancy (in particular leucemia) [15-18]. It is tempting to speculate that mediastinal germ

cell tumors and i(12p) negative TGCT may share some clinical features, namely a higher incidence of non-germ cell malignancies.

Only clinical prospective studies comparing patients with i(12p) positive and negative TGCT can tell whether there are differences in the evolution of both groups.

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This work was supported by grants GUKC 84-06 and GUKC 88-10 of the Netherlands Cancer Foundation (KWF), by the Jan Kornelis de Cock Stichting, by the Pediatric Oncology Foundation Groningen (SKOG), and by an institutional grant from the Hubrecht Laboratorium, Utrecht, The Netherlands. We are grateful to all pathologists and clinicians from the hospitals of Groningen, Assen, Leeuwarden, Winschoten, Enschede and Hoogeveen for their willingness to share their patient material with us. We also thank Menke Aikema for photographical assistance.

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CHAPTER IX

KARYOTYPING AND DNA FLOW CYTOMETRY OF A CASE OF ORCHIDOBLASTOMA

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ABSTRACT

The first karyotype of an orchidoblastoma is described. The most striking finding is the absence of the i(12p) marker chromosome, considered specific for testicular germ cell tumors of adults.

Differences between infantile and adult testicular germ cell tumors are discussed, as are features which infantile testicular germ cell tumors have in common with extragonadal germ cell tumors.

INTRODUCTION

Testicular germ cell tumors are a heterogeneous group of neoplasms with respect to histopathology [1] and age distribution of the patients [2]. There are several theories on the pathogenesis of these tumors with implications for classification [3]. Measurement of DNA ploidy and cytogenetic studies will probably shed light on the pathogenetic relationships between the different histologic subtypes of testicular GCT.

Germ cell tumors (GCT) of the infantile testis are rare neoplasms, which differ in many respects from testicular GCT of adults. We have karyotyped the first case of orchidoblastoma, and found that it lacks the i(12p) marker chromosome, which is considered specific for testicular GCT [4]. This may be yet another fundamental difference between infantile and adult testicular GCT.

CASE HISTORY

A 20 months old boy was admitted because of a three week history of scrotal swelling. The boy was born after an uneventful pregnancy and delivery. He showed no signs of congenital malformations. The left testis was normally descended, and normal on palpation. The right part of the scrotum was filled by a firm non tender mass of about 3 x 4 cm. Serum alphafetoprotein was elevated to 4400 g/l. CT-scans of abdomen and lungs and a radioisotope bone-scan revealed no metastases.

Via an inguinal approach the right testis containing the tumor was removed. After surgery the serum afphafetoprotein level normalized according to half life.

The right testis contained a tumor, measuring 3cm in diameter, which was confined to the testis. On cross section the tumor was whitish, friable, and partly surrounded by a narrow rim of testicular parenchyma. Microscopic examination showed a pure yolk sac tumor, with

characteristic Schiller Duval bodies and PAS positive globules. Alphafetoprotein was immunohistochemically demonstrated in tumor cells. The seminiferous tubules in the preserved testicular tissue did not contain carcinoma in situ.

MATERIALS AND METHODS

The tumor was submitted fresh and sterile. Tumor tissue was processed for tissue culture, karyotyping and DNA flow cytometry as described [5].

RESULTS

As the yield of metaphases was poor, only three metaphases have been fully or partially analyzed. The modal chromosome number was 76, in keeping with the results of DNA flow cytometry, which showed only one stem line with a DNA index of 1.83 (the DNA index of a diploid cell is 1, and of a tetraploid cell is 2).
Figure 1 shows the karyotype of one metaphase.

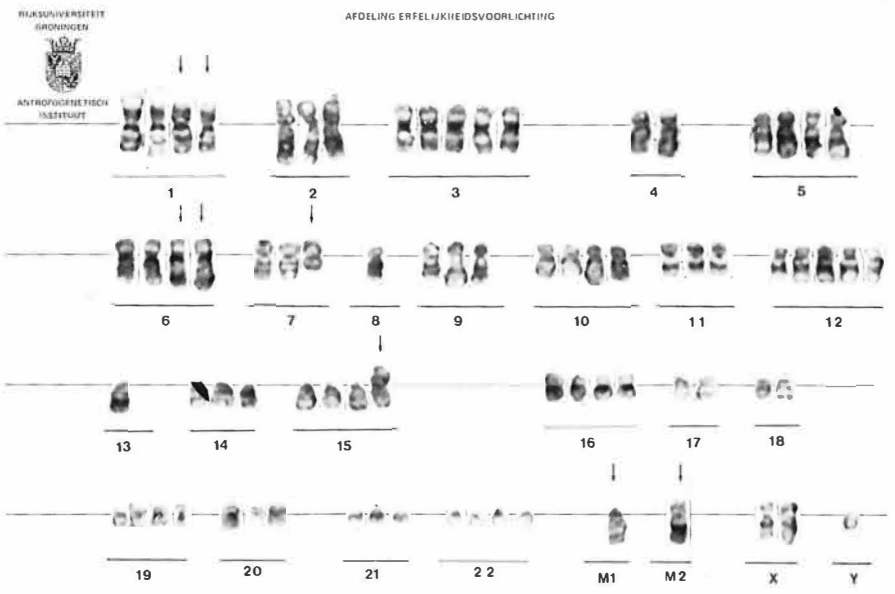

Figure 1. Karyotype of one metaphase with the following chromosomal constitution: 76,XY,+X,+2,+3,+3,+3,+5,+5,-8,+9,+10,+10,+11,+12,+12, +12?,-13,+14,+15,+16,+16,17?,+19,+19,+20,+21,+22,+22, +der(1)t(1;9)(p11 or p13;q12),+der(1)t(1;7?)(q21;q21?), +der(6)t(6;?)(q13 or q14;?),+der(6)t(6;?)(q13 or q14;?), +del(7)(q22),+der(15)t(15;15)(p11;q13),+M1,+M2(der(5)?)

Among the structural abnormalities found, we could make sure that 2 were clonal: a der(6)t(6;?)(q13 or q14;?) and a del(7)(q22) (Figure 1). All metaphases lacked the i(12p).

DISCUSSION

As compared to testicular GCT of adults, testicular GCT in the pediatric age group differ in epidemiology [2], clinical behaviour, distribution of morphological subtypes, and DNA ploidy. Most testicular GCT of infants are either pure yolk sac tumors (orchidoblastomas) or pure teratomas [6,7]. Both are rare among testicular GCT of adults [1]. The DNA ploidy of testicular GCT of adults is hypertriploid for seminomas and hypotriploid for non-seminomas, while orchidoblastomas tend to be diploid or near-tetraploid [8,9]. Testicular GCT of adults are very frequently accompanied by carcinoma in situ in the seminiferous tubules surrounding the tumor. On the contrary carcinoma in situ has not been demonstrated adjacent to orchidoblastoma [10]. With respect to risk factors, cryptorchidism is an important risk factor for testicular GCT in adults, but has not been investigated for infantile testicular GCT.

The most striking finding in the karyotype of this case of orchidoblastoma is the lack of the i(12p). In over 80% of the testicular GCT of the adult that specific marker is present [11]. Recently the same marker was found in ovarian cancer [12,13]. Therefore it is likely that the i(12p) is important in the development of gonadal germ cell tumors in general. Since that marker is not present in this case, it might be that the pathogenesis and oncogenesis of orchidoblastoma and testicular GCT of adults are different.

In a recently measured series of 12 mediastinal GCT, we found that six were diploid, five were (near)tetraploid and one was triploid (paper in preparation). Thus far we have karyotyped 2 mediastinal malignant germ cell tumors [14 and paper in preparation]. Both cases, as well as the case reported by Mann et al [15], lacked the i(12p). With respect to DNA ploidy and lack of i(12p), orchidoblastoma seems to resemble mediastinal GCT.

Our findings stress the need for further research into differences between infantile and adult testicular GCT with respect to risk factors, pathogenesis, and oncogenesis.

In view of the similarities between mediastinal GCT and orchidoblastomas, and the differences between the latter and adult

testicular GCT, it is tempting to call orchidoblastomas "extra-gonadal GCT of the testis".

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CHAPTER X

A MALIGNANT SERTOLI LEYDIG CELL TUMOR OF THE TESTIS WITH HETEROLOGOUS COMPONENTS, HAVING THE METACENTRIC GERM CELL TUMOR MARKER: i(12P)

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Submitted for publication

ABSTRACT

A malignant Sertoli Leydig cell tumor with mesenchymal heterologous elements of the testis is presented. This entity is described in the ovary, but not hitherto in the testis. Karyotyping and ploidy measurement was done of the primary tumor and of an inguinal- and lung metastases. The DNA ploidy and modal chromosome numbers were in agreement with each other in all samples. The most significant finding in the karyotype was the presence of the metacentric germ cell tumor marker 1(12p), which raises intriguing questions about the pathogenesis of the heterologous elements in the tumor.

INTRODUCTION

Sex cord stromal tumors (SCST) of the testis are rare, accounting for about 3% of all testicular neoplasms. Only a small proportion is malignant [1]. To our knowledge no cytogenetic data are available on these tumors.

We present the first description of a testicular malignant Sertoli Leydig cell tumor containing heterologous elements. Tumors of this type are known to occur in the ovary. The presence of mesenchymal heterologous tissues in ovarian Sertoli Leydig cell tumors is prognostically unfavorable [2]. Interestingly the heterologous tissue in the present case was also mesenchymal, predominantly composed of osteosarcoma.

The cytogenetic findings in this tumor [3] raise intriguing questions about its histogenesis.

CASE HISTORY

A 54 year old, white male patient presented with a large scrotal mass. On physical examination there was a tumor in the left testis. Serum alpha-fetoprotein (AFP), beta-human chorionic gonadotropin (HCG), and lactodehydrogenase (LDH) were in the normal range. There were no clinical signs of endocrine disturbances, in particular there was no gynecomastia. The steroid profile of serum and urine was normal, apart from low serum levels of testosterone. Accordingly there were low urine levels of androsterone and etiocholanolone, and elevated serum levels of follicle stimulating hormone and luteinizing hormone. Clinical staging [4] did not reveal metastatic disease. An inguinal orchidectomy was performed.

Gross examination showed a lobulated tumor, measuring 12 x 10 x 8

cm., which on cross section had a variegated appearance with interwoven, whitish, yellowish, hemorrhagic, and necrotic areas. The consistency of the tumor was firm, some areas were hard and gritty. The lesion was poorly circumscribed and invaded the paratesticular tissues in particular the epididymis. No remaining testicular parenchyma was found.

Microscopically the tumor was heterogeneous. The following components were identified: a. areas classified as malignant Leydig cell tumor, composed of polygonal cells with large eosinophilic cytoplasm, with occasional Reinke crystalloids. The nuclei were atypical and mitotic figures were regularly found (Fig. 1). b. Areas composed of smaller, atypical cuboid and fusiform cells, classified as Sertoli cells which were arranged in solid cords separated by thin fibro-vascular septa (Fig. 2). c. Areas classified as osteosarcoma, composed of large atypical, mitotically active (more than three mitoses per 10 high power fields), osteoblastic cells producing osteoid matrix which was extensively calcified. The osteosarcoma component contained large irregular spaces filled with blood, justifying the designation of telangiectatic osteosarcoma (Fig. 3). d. Areas classified as malignant giant cell tumor, composed of a dense stroma of atypical spindle cells interspersed with osteoclastic giant cells. In this component there was a high mitotic rate as well (Fig. 4). Careful screening of more then 20 slides sampled throughout the tumor did not show areas consistent with a germ cell tumor or with a mixed germ cell SCST. Immunohistochemical staining for AFP, HCG, and placental alkaline phosphatase was negative. Enzyme-histochemical staining for 3-beta-hydroxysteroiddehydrogenase, which is specific for steroid synthesizing cells [5], was positive in the neoplastic Leydig cells. Thus the tumor was classified as a malignant Sertoli Leydig cell tumor with mesenchymal heterologous elements.

Seven months after first admission the patient developed one large metastasis in the left groin, and several metastases in both lungs. Figure 5 of a chest X ray shows the very dense "bony" metastases. Blood levels of AFP, HCG and LDH were still in the normal range. Treatment consisted of a cisplatinum based multiple drug chemotherapy regimen. The inguinal metastasis was surgically removed prior to chemotherapy, the lung metastases, which progressed slowly during chemotherapy, are being removed following chemotherapy. Metastases were already removed from the right lung, and the patient awaits further surgery of the left lung.

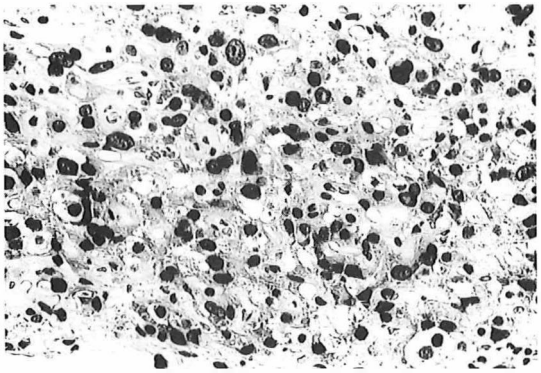

Figure 1. Primary tumor: area composed of atypical Leydig cells. (H. and E.; x 350)

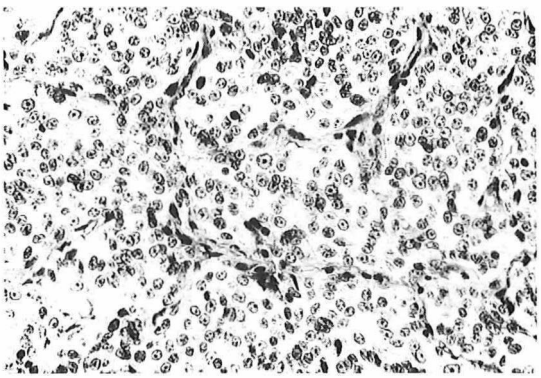

Figure 2. Primary tumor: area composed of Sertoli cells, arranged in solid cords and nests separated by thin fibrovascular septa. (H. and E.; x 350)

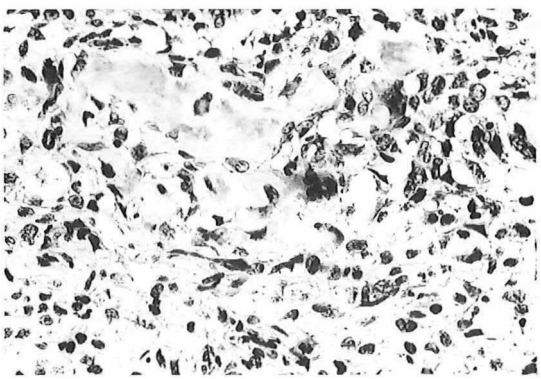

Figure 3. Primary tumor: area composed of osteosarcoma: deposition of (partly mineralized) osteoid matrix by large atypical osteoblasts. (H. and E.; x 350)

The inguinal and the lung metastases consisted predominantly of osteosarcoma. One of the lung metastases, however, had the histology of a malignant Leydig cell tumor, with unmistakable Reinke crystalloids (Fig. 6). The tumor tissue was morphologically viable without signs of regression due to chemotherapy.

MATERIALS AND METHODS

Tissue from the primary tumor and the metastases, submitted fresh and sterile, was sampled and processed for tissue culture, and DNA flow cytometry as described [6], the only difference being that flow cytometry was carried out with the FACS cell sorter instead of the Ortho ICP 22. The remaining tissue was fixed in formalin and embedded in paraffin for (immuno)histology, and DNA flow cytometry as described [6].

The DI was separately measured of the following components of the primary tumor: Leydig cell-, Sertoli cell-, osteosarcoma- and malignant giant cell tumor-components. Moreover the ploidy was measured of the inguinal metastasis, of one of the lung metastases with the histology of osteosarcoma and of the lung metastasis with the histology of malignant Leydig cell tumor. Tumor ploidy is expressed by the DNA index (DI) defined as the ratio between the modal G0,1 peak of the aneuploid population to that of the modal G0,1 peak of diploid normal cells of the samples. By definition a diploid tumor cell population thus has a DI = 1.00. The DI of two lesions was considered significantly different if the difference was $\geq$ 10%, which is more than twice the coefficient of variation which was less than 5%.

For chromosome preparations the tumor cells were harvested according to the procedures of Gibas et al. [7] after exposure to colcemid (0.5 g/ml culture medium) for five hours.

RESULTS

The results of the DNA flow cytometry, compared to the chromosome numbers of lesions that were also karyotyped, are shown in Table 1.
The DI was not significantly different in the four components of the primary tumor. The Leydig cell component had a secondary stem line with a higher DI than the main stem line. The DNA-ploidy of the primary tumor was in good agreement with the chromosome numbers in two karyotypes. The DI of the inguinal metastasis is in reasonable agreement with the numbers of chromosomes found in 13 metaphases, in particular when the

range of the chromosome counts is taken into consideration. The lung metastasis with the histology of osteosarcoma had chromosome numbers predicted from the DNA ploidy. The only DI which was significantly higher than the DI found in the other samples was measured in the lung metastasis with the histology of a malignant Leydig cell tumor. Its DI was comparable to the DI of the secondary stem line of the Leydig cell component of the primary tumor. This metastasis was not karyotyped.

Table 1 Comparison of DNA index and modal chromosome numbers of a primary malignant SCST and its metastases

Lesion	DNA index	chromosome numbers
primary tumor components		88 and 90 in two karyotypes
Leydig cell	1.95; 2.24*	(not assigned to a particular
Sertoli Leydig cell	1.86	histological component)
osteosarcoma	1.93	
giant cell tumor	1.87	
inguinal metastasis		
osteosarcoma	1.88	median 80, range 66 - 83
lung metastases		
Leydig cell	2.14	not karyotyped
osteosarcoma	1.87	median 83, range 76-86

*secondary stem line

The results of karyotyping were more extensively published elsewhere [3]. A brief description of the most significant findings is given here. From the primary tumor only 2 abnormal metaphases (with 88 and 90 chromosomes) were obtained, but none was analyzable. From the inguinal metastasis 13 metaphases were analyzed. Chromosome counts ranged from 66 to 83, with a median of 80. Figure 7 shows a representative karyotype of the inguinal metastasis, with the following chromosomal constitution:

82,X,-Y,+4,+4,-6,-8,-9,+14,-15,-17,-17,+19,+19,+20,+20,+20,+20,
del(1)(q11),del(1)(q21),+der(1)t(dup(1);?)(?::1p36-->q44::q44-->q12),
+der(2)t(ctb(2)(p24);?)(q11 or q12;?),+der(2)t(2;7)(q11;q11),
+del(3)(q21),+der(5)t(5;?)(p14;?),+der(6)t(6;?)(q11;?),
+der(8)t(8;?)(p23;?),+der(9)?,del(10)(q21),+del(11)(q11),
+der(12)t(12;?)(q11;?),+der(12)t(11?;12)(q13?;p13),+del(12)(q13),
+i(12p),+der dic(13)t(8;13)(q11;p13),+der(14)t(14;?)(q31;?),
+der dic(14)t(14;?)(p11;?),+der(15)t(15;?)(q26;?),
+der(16)t(16;?)(p13.3;?),+del(18)(p11),+der(21)t(21;?)(p12;?),
+i(del(22)(q13)),+i(del(22)(q13)),+M1,+M1,+M2,+M3(1q12-->q41::?),
+M4(der(5)?),+M5,+M6(der(12)),+M7,+M8,+M9,+M10(der(12p)).

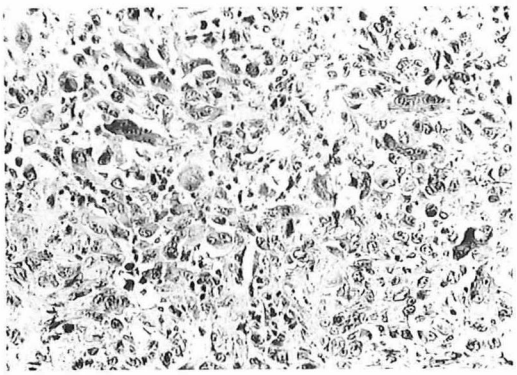

Figure 4. Primary tumor: area resembling malignant giant cell tumor: background of atypical spindle and epithelioid cells inter-spersed with large osteoclastic giant cells. (H. and E.;x140)

Figure 5. Chest X ray showing multiple dense coin lesions in both lungs

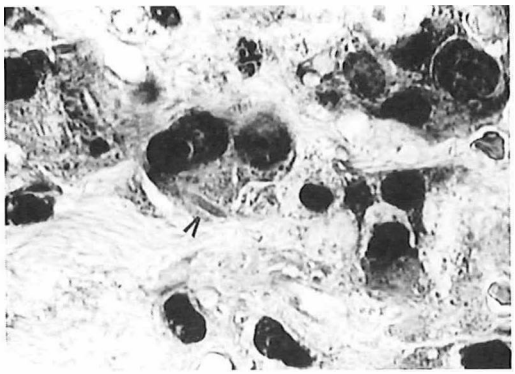

Figure 6. Histology of one of the lung metastases: tumor tissue exclusively composed of atypical Leydig cells. The arrow head points to a Reinke crystalloid. (H. and E.: x 560)

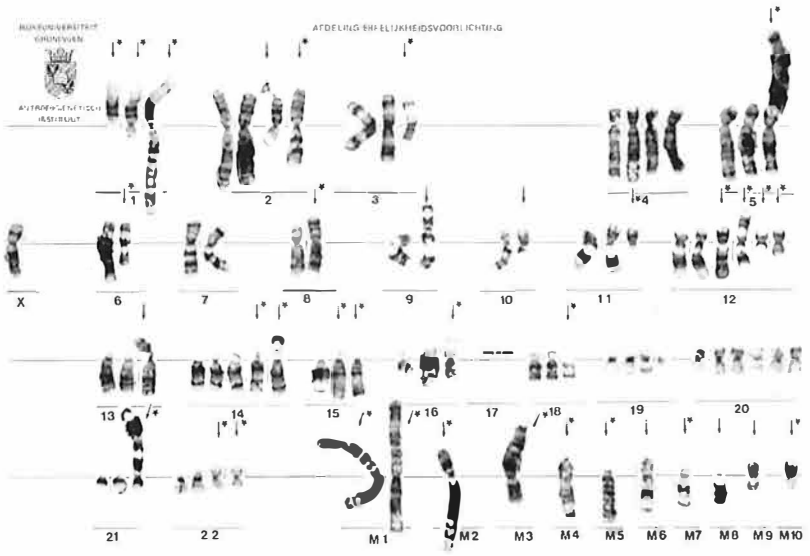

Figure 7. Karyotype of the inguinal metastasis showing the metacentric germ cell tumor marker i(12p)

DISCUSSION

The tumor reported here must be very rare. To our knowledge no such neoplasm in the testis has been described . Nevertheless the tumor can be histologically classified with confidence as a Sertoli Leydig cell tumor with heterologous components because similar neoplasms have been reported in the ovary [2], and most SCST of the ovary have their counterparts in the testis [1].

A histological differential diagnosis which should be considered is a teratoma with malignant transformation [8]. The existence of this entity in the testis is questioned by Talerman [9], but recent papers dealing with residual teratoma after chemotherapy recognize secondary non-germ cell malignancies along somatic differentiation lineages in primary and metastatic non-seminomas [10-13]. The absence of germ cell tumor elements, however, and the presence of a Sertoli Leydig cell component argue against teratoma with malignant transformation.

Another differential diagnosis to be considered is a mixed germ cell SCST. These tumors essentially consist of neoplastic sex cord stromal elements with interspersed non-neoplastic germ cells. To date no association or overgrowth of seminoma or other neoplastic germ cell elements has been described [9]. Moreover, tumors of this type in the

testis are not known to metastasize [9].

The most interesting finding in the karyotype is the i(12p) marker chromosome, considered specific for germ cell tumors of the testis [14]. In view of the complex karyotype it is conceivable that the marker is a secondary chromosomal change. It is tempting to speculate, however, that the marker does indicate a germ cell origin of the heterologous components. In that case we would be dealing with the hitherto unrecognized entity of a mixed germ cell SCST in which neoplastic outgrowth of the germ cell component did take place in the form of teratoma with malignant transformation. The results of measurement of DNA ploidy give no clue as to the origin of the heterologous components. The different histological components in the primary tumor, and the metastases with the histology of osteosarcoma in the groin and in the lung all had a very similar, hypotetraploid DI. Most likely, in the course of tumor progression, polyploidization has taken place with subsequent loss of chromosomes. The lung metastasis with the histology of a malignant Leydig cell tumor belongs to a clone with a different DI, which may have been represented by the secondary stem line of the Leydig cell component of the primary tumor.

In the only published description of a karyotype of a SCST, an ovarian granulosa cell tumor, no i(12p) was found. The karyotype showed similarity with karyotypes of carcinomas of the ovary [15].

The i(12)p marker chromosome is found in more than 80% of all testicular germ cell tumors [16-19], and was recently described in two ovarian germ cell tumors classified as dysgerminomas [20, 21]. The only gonadal non-germ cell tumor in which it was reported was a mixed Muellerian tumor [21], a highly malignant tumor which is characterized by the presence of mesenchymal heterologous elements [22]. The most obvious common denominator between the present tumor, germ cell tumors and mixed Muellerian tumors is pluripotency. It might be speculated that if the i(12p) would occur in a differentiated cell, this might lead to loss of restriction of differentiation and hence pluripotency. Alternatively, it seems conceivable that the occurrence of the i(12p) in an undifferentiated pluripotent stem cell can result in a malignant pluripotent cell.

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CHAPTER XI

PATHOGENESIS AND ONCOGENESIS OF TESTICULAR GERM CELL TUMORS. CYTOGENETIC SUPPORT FOR A UNIFYING MODEL

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INTRODUCTION

Testicular germ cell tumors of adults can be divided clinically and morphologically in two main entities: seminomas and nonseminomas [1-3].

Essentially there are two theories about the pathogenetic relationship between seminomas and nonseminomas [4-7]. One favors independent origins of the subtypes via carcinoma in situ (CIS) [4,5]. The other suggests a single origin for the tumors with seminoma as a stage after in situ carcinoma through which all testicular germ cell tumors progress [6,7].

Support for the latter view comes from our [8,9] and other [10] studies about the cellular DNA content of testicular germ cell tumors, and from cytogenetic studies of seminomas [11,12], nonseminomas [13-15], and a combined germ cell tumor of the testis [16] in which both the seminoma and the nonseminoma components shared two marker chromosomes. Further support comes from phenotypic similarity between carcinoma in situ cells and seminoma cells [17,18] and from a study of a testicular tumor with borderline histology between seminoma and embryonal carcinoma [19].

A cytogenetic comparison of primary seminomas and nonseminomas, and of primary nonseminomas and mature residual teratomas following polychemotherapy may help to clarify the pathogenesis of testicular germ cell tumors, and suggest which chromosomes are important for malignancy and tumor progression, and which for tumor suppression. Chromosomes important for tumor suppression might also be essential for differentiation [20-28].

We recently described our chromosomal findings in seminomas [12], primary nonseminomas [15], and mature residual teratomas following intensive chemotherapy [29]. Here we present a statistical analysis of the data obtained for these various subtypes of testicular germ cell tumors, and discuss its pathogenetic implications.

MATERIALS AND METHODS

For this comparative study cytogenetic data from 10 primary seminomas [12], 14 primary nonseminomas [15], and 13 residual teratomas following chemotherapy [29] were analyzed as follows:

a. Per patient we determined separately the modal number of short and long arms present for each chromosome, except the acrocentric chromosomes. Parts of chromosomal arms involved in structural abnormalities were

entered as whole arms if representing 50% of the total arm length. Smaller segments were disregarded. The data obtained were subjected to an analysis of variance in order to answer three main questions: **is the average number of short arms significantly different from the average number of long arms for specific chromosomes? Does the average number of short arms plus long arms per chromosome show significant differences between the three groups? Is there a characteristic pattern in the proportion of the different chromosomes present for each group of tumors?**

Data concerning the acrocentric chromosomes were analyzed in a similar way, taking into account the long arms only.

b. The average number of copies of i(12p) found in the three groups of tumors was analyzed by Kruskall-Wallis analysis of variance [30] to test for the presence of differences between groups, followed by comparisons between groups using the one-sided Mann Whitney U test [30].

Full details about the statistical methods used, and their respective results can be found in the Appendix.

RESULTS AND DISCUSSION

Figure 1 shows the average number of short and long arms present for each chromosome in the whole series of tumors (i.e. seminomas plus nonseminomas plus mature residual teratomas).

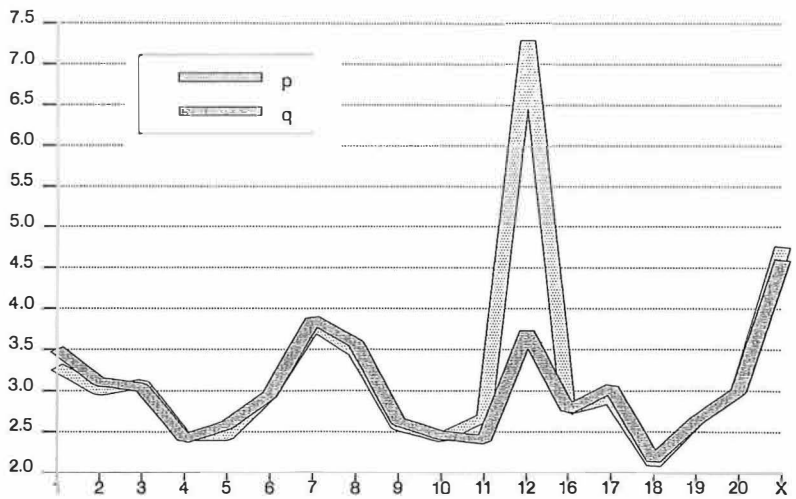

Figure 1. Average number of short arms and long arms per specific chromosome in the 3 groups (seminomas, primary nonseminomas, and residual teratomas)

The average number of short arms plus long arms per specific chromosome in each group of tumors is shown in Figs. 2 and 3 for the non-acrocentric chromosomes and the acrocentric chromosomes, respectively.

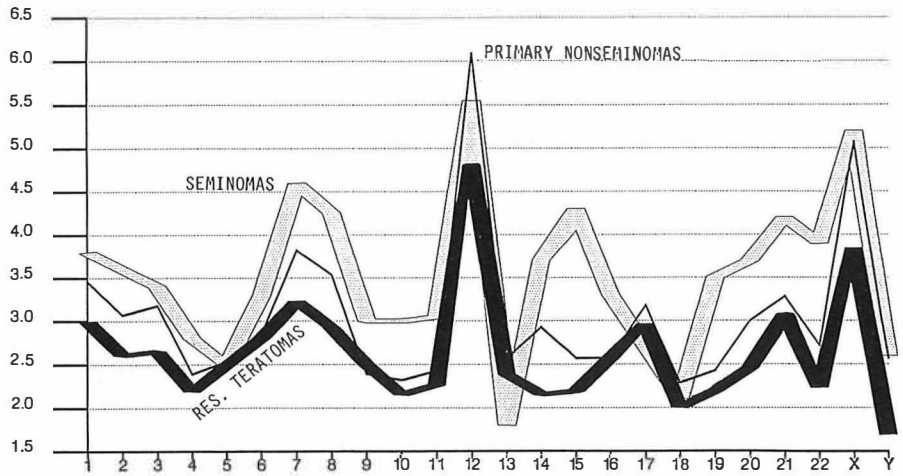

Figure 2. Average number of short arms plus long arms per specific chromosome in each group of tumors (only non-acrocentric chrs.)

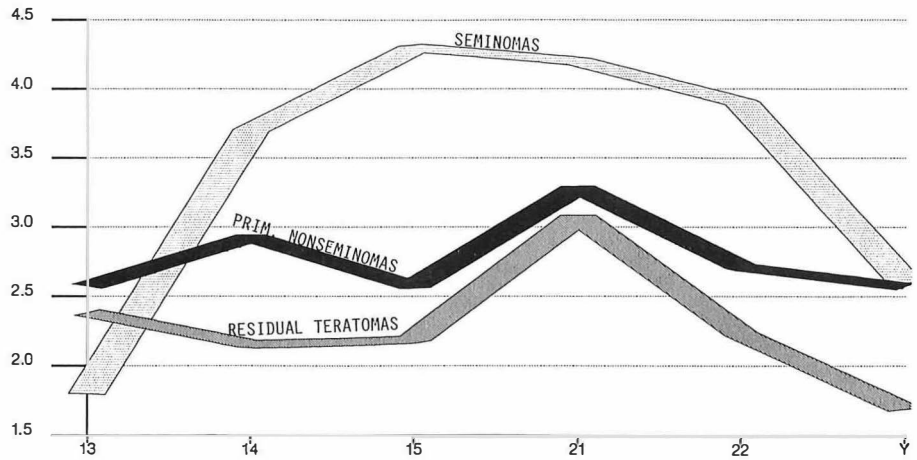

Figure 3. Average number of long arms of the acrocentric chromosomes per specific chromosome in each group of tumors

Figure 4 displays the data presented in Figs. 2 and 3 in the form of an histogram.

Relationship between seminomas and nonseminomas

The comparison of the average number of copies of the different chromosomes in seminomas and nonseminomas showed a striking similarity of the relative proportions of the non-acrocentric chromosomes (Fig. 2). We consider this finding (hardly conceivable in unrelated tumors) a convincing argument in favor of their common origin. Tumor progression in testicular germ cell tumors apparently goes from high to lower number of chromosomes [15]. Since nonseminomas are more aggressive than seminomas (see [31] for review), it is conceivable that the chromosomes present in seminomas in higher numbers than in nonseminomas are important for tumor suppression and normal differentiation [20-28]. In this respect, it is of interest that the relative proportion of the acrocentric chromosomes in seminomas is significantly different from the one observed in nonseminomas (Fig. 3).

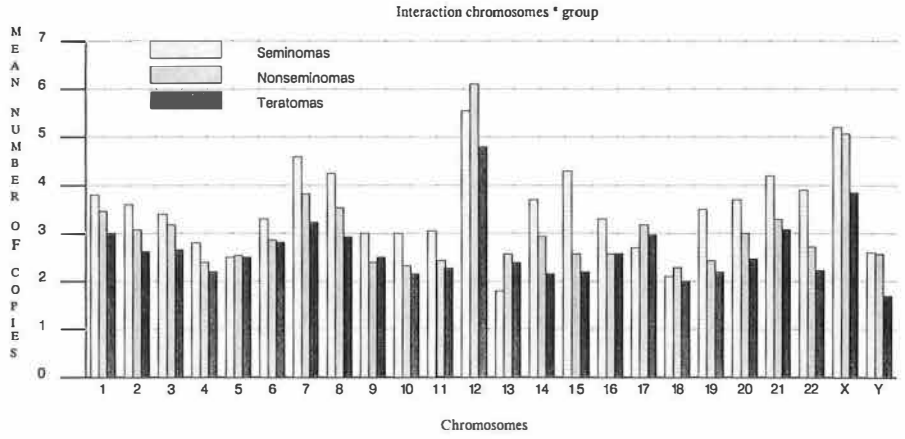

Figure 4. Data used for Figs. 2 and 3 is displayed here in the form of an histogram

As compared to seminomas, some acrocentric chromosomes (especially #15 and #22) have significantly less copies in nonseminomas. Thus, it might be speculated that loss of these chromosomes is crucial for a seminoma stage cell to become a nonseminoma, possibly because of loss of genes important for sperm cell differentiation. Chromosomes consistently

underrepresented in seminomas and nonseminomas (mainly #5, #11, #13, and #18) may contain genes important for tumor suppression in general and/or normal germ cell differentiation. Chromosomes present in higher numbers in nonseminomas (mainly #7, #8, and #12) may be responsible for a more malignant development.

Relationship between primary nonseminomas and residual mature teratomas following chemotherapy

Our comparison of the numbers of the different chromosomes in primary nonseminomas and residual teratomas revealed significant differences in the relative representation of the non-acrocentric chromosomes (Fig. 2).

Since residual teratomas have only slightly less chromosomes than primary nonseminomas, it is likely that the critical difference between them resides in changes in the balance between chromosomes (hence genes) allowing and suppressing differentiation, rather than in heavy chromosomal loss. This would be in keeping with our cytogenetic findings of less abnormal karyotypes in residual teratomas [29,34] as compared to primary nonseminomas [15]. The new balance of genes in residual teratomas would be in favor of genes with tumor suppressing and differentiation regulating properties. The finding of a lower average number of #12 (especially of i(12p)), as compared to primary nonseminomas, points to a possible correlation between the number of copies of that marker and a more aggressive behaviour.

The results presented here may best be considered to support the unifying model of pathogenesis of testicular germ cell tumors of Ewing [32] and Friedman [33], according to which seminomas and nonseminomas have a common origin. Our studies on chromosomes [12,15,29] and ploidy of testicular tumors [8,9] strongly suggest that the progression of these malignancies correlates with a net decrease in the number of chromosomes. Loss of chromosomes #15 and #22 may be particularly critical in the oncogenesis of nonseminomas. A change in gene balance rather than gross differences in chromosome numbers seem consistent with the observed cytogenetic differences between primary nonseminomas and residual teratomas.

APPENDIX

Statistical analysis

The number of copies of the p and the q arm for the non-acrocentric chromosomes in three types of testis tumors, seminomas,

nonseminomas, and teratomas was taken as the dependent variable. Grouping factors were the type of the tumor, the p or q observation, and the chromosome number. The p or q observations are in most cases dependent, because one intact chromosome leads to a count for the p and for the q part. The data for the non-acrocentric chromosomes were analyzed as a 3x2x18 factorial design, with the number of observations dependent on the type of tumor. The acrocentric chromosomes were analyzed in a 3x6 factorial design (tumorsxchromosomes) The data can be described with a simple model, which explains 44.8% of the total variance. The model is:

Number of copies observed=
constant+
chromosome dependent value+
tumor type dependent value+
value dependent on p or q arm (for the non-acrocentric chromosomes)+
interaction between chromosome and tumor type.

The tables for the analysis of variance of this model, as computed by the SYSTAT [35] program are shown below (Tables 1 and 2).

Table 1 - Analysis of variance for the non-acrocentric chromosomes

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	Probability
Chromosomes	716.677	17	42.157	37.464	0.000
tumor type	106.287	2	53.144	47.228	0.000
p or q part	9.586	1	9.586	8.519	0.004
Chromosomes* tumor type	68.525	34	2.015	1.791	0.004
Chromosomes* p or q part	229.589	17	13.505	12.002	0.000
ERROR	1417.836	1260	1.125		

Most of the variance is explained by differences in chromosome counts per chromosome and per tumor type. The interaction between chromosomes and tumor type means that the relative proportions in which chromosomes are found, depends on the tumor type. An analysis of the differences in chromosome counts between pairs of tumors reveals that there is a significant difference in pattern between the teratomas on one hand and the seminomas and nonseminomas on the other hand. The interaction between the p or q classification and the chromosome classification is entirely due to the fact that 12p arms are very frequent in comparison to 12q arms (Fig. 1). This is for a large part the same finding as the difference of the i(12p) chromosome between tumor groups, as analyzed with non-parametrical statistics. Figure 2 shows the main effect due to the type of tumor: on average most copies of p and q are found for the seminomas, followed by the nonseminomas, and then by the teratomas. This applies as well to the acrocentric chromosomes, as is shown in Fig. 3. The differences dependent on the chromosome number (main effect) can be seen in both Figs. 1 and 2. Chromosomes 7 and 12 are present in higher numbers than the others. The

significant interaction between chromosome number and tumor type is mainly explained by noting that the ratio of the number of copies of chromosome 12 compared to chromosome 7 depends strongly on the tumor type. In quantitative terms this interaction is the least important effect, as the F ratio is only 1.791 compared with more than 8.519 for the other effects. For the acrocentric chromosome the interaction between chromosomes and tumor type is due to the relatively higher number of copies of the chromosomes #14, #15, #21 and #22, compared to chromosomes #13 and Y, for the seminomas in contrast with that in the teratomas and nonseminomas.

Table 2 - Analysis of variance for the acrocentric chromosomes

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	Probability
Chromosomes	126.386	5	25.277	24.706	0.000
Tumor type	37.680	2	18.840	18.414	0.000
Chromosomes* Tumor type	32.912	10	3.291	3.217	0.001
ERROR	208.719	204	1.023		

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SUMMARY

Testicular germ cell tumors of adults can be divided both clinically and morphologically in two distinct entities, seminoma and nonseminoma. In about 20% of germ cell tumors seminomas and nonseminomas coexist. Seminomas are less aggressive than nonseminomas and combined tumors. It is presently accepted that these malignancies arise from a dysplastic precursor cell via carcinoma in situ. However, it is still controversial whether seminomas and nonseminomas have a common or independent origin. One pathogenetic model suggests that seminomas and nonseminomas are independently derived from separate carcinoma in situ lesions, whereas another theory assumes that all germ cell tumors (with the possible exception of spermatocytic seminoma) have a single origin with seminoma as a stage after carcinoma in situ.

The genetic mechanisms involved in the oncogenesis and pathogenesis of testicular germ cell tumors are still poorly understood.

The characterization of the different subtypes of primary testicular germ cell tumors on the grounds of their ploidy is presented in chapter 2. Using DNA flow cytometry, a significantly different median DI was found for orchidoblastomas, seminomas, and nonseminomas, of respectively: 1.91, 1.66 and 1.43. The seminoma and nonseminoma components of combined tumors (n=16) had a significantly different median DI of 1.61 and 1.40 respectively. Three of the 10 orchidoblastomas were diploid, compared to only one of the 72 testicular tumors of adults.

The cytogenetical confirmation of these findings is shown in chapters 3 and 4. The clustering of germ cell tumors in the triploid range suggests that fusion of a post-meiotic haploid cell with a diploid cell, or tetraploidization followed by chromosomal loss are probably early events in the oncogenesis of testicular germ cell tumors of adults. However, seminomas with chromosome numbers higher than 70 are not unusual, whereas hypertriploid nonseminomas are extremely rare. As noted in chapters 3 and 4, both in seminomas and nonseminomas specific chromosomes are consistently underrepresented (e.g., #11, #13, #18, and

Y), whereas other chromosomes were consistently overrepresented (e.g., #7, #8, #12, and X). It is conceivable that the chromosomes consistently underrepresented may contain genes important for normal germ cell differentiation and/or with tumor suppressing properties. Chromosomes consistently overrepresented may contain genes responsible for a more malignant development. The average number of copies of the i(12p) is significantly lower in seminomas than in nonseminomas. Chapter 5 describes the cytogenetical findings in a combined germ cell tumor of the testis, theoretically a good model to study the possible relationship between seminomas and nonseminomas. The only structural abnormality in common between the seminoma and the nonseminoma components was the i(12p). Since this marker is found in over 80% of all testicular germ cell tumors, the presence of the i(12p) in both components does not allow per se the conclusion that they have a common origin. A simple technical approach is described, which gives the possibility of a separate cytogenetical study of the seminoma and nonseminoma components in combined tumors.

Chapter 6 describes the chromosomal changes found in a series of mature residual teratomas following chemotherapy. A cytogenetic comparison between residual teratomas and primary nonseminomas showed that residual teratomas have a lower average number of structural abnormalities (including the i(12p)), a lower number of copies of #7, #8, #12, #14, X, and Y. This finding confirms the hypothesis that residual teratomas are the result of chemotherapeutic selection of less malignant (less abnormal) clones from the primary tumor. In chapter 7 similar findings were noted in the cytogenetical comparison of the primary nonseminoma and the residual teratoma following chemotherapy in the same patient.

Chapter 8 points to the existence of i(12p) negative testicular germ cell tumors, providing some preliminary indication for a different clinical evolution and prognosis, as compared to germ cell tumors with the referred marker.

Chapter 9 presents the first chromosomal study of a case of orchidoblastoma, pointing out the similarities between infantile testicular germ cell tumors and extragonadal germ cell tumors, and the differences between the former and germ cell tumors of the adult testis.

Chapter 10 discusses the implications of the finding of an i(12p) in a Leydig/Sertoli cell tumor of the testis, which is a non-germ cell

malignancy.

Chapter 11 presents a statistical processing of all cytogenetical data obtained in primary seminomas and nonseminomas, as well as in residual teratomas following chemotherapy. A striking similarity between the relative proportion of the non-acrocentric chromosomes in seminomas and primary nonseminomas was noted. This clearly points to their pathogenetic relationship, lending support to the model suggesting that seminomas and nonseminomas have a common origin. As compared to seminomas, nonseminomas show a remarkable decrease in the number of copies of #15 and #22. This finding suggests that #15 and #22 may contain genes crucial for sperm cell differentiation. The comparison between primary nonseminomas and residual teratomas shows different relative proportions of the non-acrocentric chromosomes, suggesting that the critical difference between residual teratomas and primary nonseminomas resides in changes in the balance of chromosomes favoring tumor suppression and differentiation, rather than gross chromosomal loss. The overall comparison of seminomas, primary nonseminomas, and residual teratomas shows that tumor progression of testicular germ cells of adults is accompanied by a net loss of chromosomes. Moreover, it appeared that the least aggressive tumors (seminomas and residual teratomas) have less copies of specific chromosomes, namely #7, #8, and Y, as compared to the primary nonseminomas (more aggressive).

SAMENVATTING EN PERSPECTIEF

Kwaadaardige kiemceltumoren van de testis kunnen op grond van het microscopische beeld in twee groepen worden verdeeld: seminomen (ca 60%) en nonseminomen (40%). Bovendien is er een kleine groep tumoren met zowel een seminoom als een nonseminoom component, de zgn. gecombineerde tumoren. Seminomen zijn minder kwaadaardig en hebben een betere prognose dan nonseminomen. Omtrent de pathogenese en oncogenese van kiemceltumoren van de testis is weinig bekend. Er zijn in essentie twee verschillende theorieën omtrent de pathogenetische relatie tussen seminomen en nonseminomen.

De eerste theorie gaat er vanuit dat seminomen en nonseminomen onafhankelijk van elkaar ontstaan uit getransformeerde (dysplastische) intratubulaire kiemcellen via carcinoma in situ, de tweede dat seminomen en nonseminomen een gemeenschappelijke oorsprong hebben en dat de progressie van nonseminomen verloopt via een seminoom-voorstadium.

Omtrent de genetische mechanismen die betrokken zijn bij de oncogenese en pathogenese van kiemceltumoren van de testis is eveneens weinig bekend. De DNA-ploidie van de verschillende subtypen primaire kiemceltumoren wordt gerapporteerd in hoofdstuk 2. Gebruik makend van DNA flow cytometrie werd een significant verschillende mediane DNA index gevonden voor orchidoblastomen, seminomen en nonseminomen van respectievelijk: 1.91, 1.66 en 1.43. De seminoom en nonseminoom component van gecombineerde tumoren hadden een significant verschillende mediane DNA index van respectievelijk 1.61 en 1.41. Drie van de tien orchidoblastomen waren diploid in tegenstelling tot slechts één van 72 testiculaire kiemceltumoren bij volwassenen. In de hoofdstukken 3 en 4 is aangetoond dat in overeenstemming met de DNA index het chromosomenaantal in seminomen hoger is dan in nonseminomen.

De omstreeks triploide chromosomen aantallen van kiemceltumoren suggereren dat fusie van diploide met diploide of met post-meiotische haploide cellen of polyploidisatie, gevolgd door chromosomenverlies waarschijnlijk vroege gebeurtenissen zijn in de oncogenese van testiculaire kiemceltumoren van volwassenen.

Zoals weergegeven in de hoofdstukken 3 en 4 zijn zowel in seminomen als in nonseminomen bepaalde chromosomen, met name de chromosomen 11, 13, 18 en het Y chromosoom, consistent ondervertegenwoordigd en andere, met name de chromosomen 7, 8, 12 en het X chromosoom, consistent oververtegenwoordigd.
Het is aannemelijk dat de chromosomen die ondervertegenwoordigd zijn genen bevatten belangrijk voor normale kiemcel- differentiatie en/of genen met tumorsuppressie eigenschappen.
De oververtegenwoordigde chromosomen bevatten waarschijnlijk genen, verantwoordelijk voor een meer maligne ontwikkeling.

In iets meer dan 80% van alle chromosomaal geanalyseerde kiemceltumoren werd een specifiek afwijkend chromosoom, het i(12p) chromosoom gevonden. Het gemiddelde aantal exemplaren van dit i(12p) chromosoom is significant hoger in nonseminomen dan in seminomen. Dit duidt mogelijk op een positieve correlatie tussen het aantal exemplaren van het i(12p) chromosoom en maligniteit.

In hoofdstuk 5 wordt het chromosomenpatroon van een gecombineerde kiemceltumor van de testis beschreven. Dit type tumor is het ideale model om de mogelijke relatie tussen seminomen en nonseminomen te bestuderen. De enige structurele chromosomale afwijking die de seminoom en nonseminoom component gemeenschappelijk hadden was de i(12p). Omdat dit afwijkende chromosoom in meer dan 80% van alle testistumoren wordt gevonden mag uit de aanwezigheid van de i(12p) in beide componenten niet geconcludeerd worden dat ze een gemeenschappelijke oorsprong hebben. In genoemd hoofdstuk wordt een eenvoudige technische benadering beschreven die de mogelijkheid biedt de beide componenten van een gecombineerde tumor cytogenetisch te bestuderen.

In hoofdstuk 6 worden de chromosomale afwijkingen beschreven die gevonden werden in een serie residuale teratomen na chemotherapie. Een vergelijking van de cytogenetische bevindingen in residuale teratomen en primaire nonseminomen toonde dat residuale teratomen gemiddeld een geringer aantal structurele afwijkingen hebben - met name ook een kleiner aantal exemplaren i(12p) - dan primaire nonseminomen en verder minder exemplaren van de chromosomen 7, 8, 12, 14, X en Y. Deze resultaten bevestigen de hypothese dat residuale teratomen het gevolg zijn van chemotherapeutische selectie van minder maligne (minder abnormale) clones uit de primaire tumor. In hoofdstuk 7 worden overeen-

komstige bevindingen gerapporteerd bij de vergelijking van het primaire nonseminoom en het residuale teratoom na chemotherapie bij dezelfde patiënt.

In hoofdstuk 8 wordt gewezen op het bestaan van testiculaire kiemceltumoren zonder i(12p). Argumenten worden aangevoerd die er op zouden kunnen wijzen dat deze tumoren qua biologisch gedrag en prognose verschillen van de kiemceltumoren met i(12p).

In hoofdstuk 9 worden de resultaten beschreven van de eerste karyotypering van een orchidoblastoom. In deze tumor werd geen i(12p) gevonden. Deze en andere resultaten en gegevens uit de literatuur wijzen op een grotere overeenkomst tussen orchidoblastoom en extragonadale kiemceltumoren dan tussen orchidoblastoom en kiemceltumoren van de volwassen testis. Dit leidt tot de paradoxale hypothese dat het orchidoblastoom de extragonadale kiemceltumor van de testis is.

In hoofdstuk 10 wordt de opmerkelijke bevinding beschreven van een i(12p) in een niet-kiemceltumor van de testis: een maligne Sertoli Leydig cel tumor met een mesenchymale heterologe component in de vorm van osteosarcoom. De histogenese van de heterologe component wordt besproken in het licht van de chromosomale afwijkingen.

In hoofdstuk 11 tenslotte, wordt de vergelijkende statistische bewerking gepresenteerd van de resultaten van het chromosomale onderzoek van primaire seminomen, primaire nonseminomen en residuale mature teratomen na chemotherapie. Er blijkt een opvallende consistentie te bestaan met betrekking tot de relatieve verhouding van de niet-acrocentrische chromosomen bij primaire nonseminomen en seminomen. Dit resultaat is een duidelijke aanwijzing voor de pathogenetische verwantschap tussen de twee tumortypen en steunt het model van de gemeenschappelijke oorsprong. De chromosomen 15 en 22 komen in nonseminomen veel minder vaak voor dan in seminomen. Deze bevinding suggereert dat op de chromosomen 15 en 22 genen gelegen zijn die een cruciale rol spelen bij spermatocytaire differentiatie. Vergelijking van primaire nonseminomen en residuale teratomen toont een verschil met betrekking tot de relatieve verhoudingen van de niet-acrocentrische chromosomen. Dit suggereert dat het essentiële verschil tussen residuale teratomen en primaire nonseminomen gelegen is in subtiele verschillen in het evenwicht tussen chromosomen die tumorsuppressie en differentiatie bevorderen enerzijds en chromosomen die tumorgroei bevorderen anderzijds. Er is geen sprake van een belangrijk verschil in aantal chromoso-

men tussen de beide tumortypen. Het vergelijkende onderzoek waarin seminomen, primaire nonseminomen en residuale teratomen betrokken werden, leert dat tumorprogressie van testiculaire kiemceltumoren van volwassenen gepaard gaat met een netto verlies van chromosomen. Bovendien blijkt dat dit verlies niet willekeurig is. De minst agressieve tumoren (seminomen en residuale teratomen) bezitten minder exemplaren van bepaalde chromosomen, namelijk: 7, 8 en Y dan de gemiddeld meer agressieve primaire nonseminomen.

De resultaten van deze studie geven belangrijke aanknopingspunten voor verder onderzoek. In de eerste plaats geven de cytogenetische bevindingen duidelijke aanwijzingen omtrent de gebieden in het genoom waar moleculair genetisch onderzoek op moet worden gericht. In de tweede plaats hebben de resultaten geleid tot hypothesen met betrekking tot de rol van bepaalde chromosomen in tumorsuppressie en differentiatie (spermatocytair en somatisch) en van andere chromosomen in tumorpromotie. Deze hypothesen zijn in principe te toetsen door in vitro de chromosomale samenstelling van tumorcellijnen te manipuleren, zodat de balans van de chromosomen ten gunste van een bepaalde richting uitvalt. Het uiteindelijke resultaat van zulke experimenten zou diagnostische en therapeutische betekenis kunnen hebben. Diagnostisch, omdat het chromosomenpatroon prognostische betekenis heeft. Therapeutisch, omdat de mogelijkheid van inductie van differentiatie op grond van het chromosomenpatroon te voorspellen zou kunnen zijn.